Synthetic domestic wastewater sludge as electron donor in the reduction of sulphate and treatment of acid mine drainage

by

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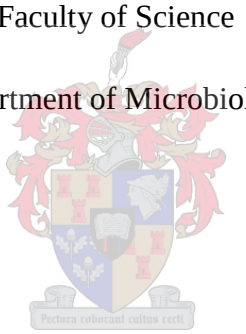

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DECLARATION

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Summary

Acid mine drainage (AMD) is wastewater generated by mine and industrial activity with typically high heavy metal and sulphur content potentially resulting in toxic wastewater upon exposure to dissolved oxygen, water and micro-organisms. Due to the hazardous consequences of untreated AMD, treatment methods such as semi-passive biotic treatments, including constructive wetlands and microbial bioreactors were developed. Microbial bioreactors rely on suitable carbon sources such as ethanol, grasses and manure and the creation of anaerobic conditions for the reduction of sulphate, chemical oxidizable organic matter (COD) and to neutralise pH. Domestic wastewater sludge has also been identified as an economical and readily available carbon source that allows the treatment of both AMD and domestic wastewater.

A synthetic medium simulating the COD and the biological degradable organic matter (BOD) of domestic wastewater sludge was formulated to exclude variations in the evaluation of domestic wastewater sludge as carbon source in the treatment of AMD. Firstly the BOD and COD of anaerobic domestic wastewater sludge was determined and used as parameters in the formulation of the synthetic medium. A ratio of 1:1 AMD: synthetic domestic wastewater sludge (SDWWS) was the optimum ratio in terms of sulphate and COD removal.

Secondly, medical drip bags were used as anaerobic bioreactors to determine the microbial diversity in AMD treated with SDWWS using different variables. Data analyses from next generation sequencing showed that *Chlorobium* spp. dominated the 90 d pioneer trials at relative percentages of 68 % and 76 %. Transmission electron microscopy (TEM) images and the bright green colour of the liquid contents confirmed the data analyses. Sulphates and COD were removed at > 98 % and > 85 %, respectively.

A shorter incubation time was investigated in the 30 d pioneer trial. *Chlorobium* spp. was dominant, followed by *Magnetospirillum* spp. and *Ornithobacterium* spp. The liquid content changed to a dark brown colour. COD and sulphate concentrations were reduced by 60.8 % and 96 %, respectively, within 26 d, after which a plateau was reached. The effect of an established biofilm in the bioreactors showed that *Chlorobium* spp. also dominated approximately 62 %, in comparison to the 36 % in the 30 d pioneer trial. A sulphate and COD reduction of 96 % and 58 %, respectively, was obtained within 26 d and the liquid content

was the same colour as in the 30 d pioneer trial. It is possible that brown Green sulphur bacteria were present. Therefore, although *Chlorobium* spp. was present at a higher percentage as in the 30 d pioneer trial, the removal of COD and sulphate was similar. During the 30 d trials a white precipitant formed at the top of the bioreactors, consisting primarily of sulphate and carbon that was also indicative of the presence of *Chlorobium* spp. Incubation at reduced temperature reduced sulphates by only 10 % and COD by 12 % after 17 d, followed by a plateau. *Ornithobacterium* spp. dominated in the first trial and *Magnetospirillum* spp. in the second trial.

Opsomming

Suur mynwater (SMW) is afvalwater wat deur die myn- en industriële bedryf gegenereer word en bevat kenmerklik hoë konsentrasies swaar metale en swawel wat potensieel in toksiese afvalwater omskep kan word indien blootgestel aan opgelosde suurstof, water en mikro-organismes. Die skadelike gevolge wat blootstelling aan onbehandelde SMW mag hê, het gelei tot semi-passiewe behandelinge wat vleilande en mikrobiese bioreaktors insluit. Mikrobiese bioreaktore maak staat op n geskikte koolstofbron soos etanol, grasse en bemesting en die skep van ‘n anaerobiese omgewing vir die verwydering van sulfate en chemies oksideerbare organiese material (CSB), asook die neutralisering van pH. Huishoudelike afvalwaterslyk is ook uitgewys as ‘n ekonomies geskikte en algemeen beskikbare koolstofbron wat die behandeling van beide SMW en huishoudelike afvalwater toelaat.

‘n Sintetiese medium wat die CSB en biologies afbreekbare organiese materiaal (BSB) van huishoudelike afvalwater slyk naboots is geformuleer om die variasies in die evaluasie van huishoudelike afvalwater slyk as koolstofbron vir die behandeling van SMW, uit te sluit. Eerstens is die BSB en die CSB van huishoudelike afvalwater slyk bepaal en gebruik as n maatstaf vir die formulering van die sintetiese medium. ‘n Verhouding van 1:1 sintetiese huishoudelike afvalwater slyk (SDWWS) en SMW is optimaal ratio i.t.v. die verwydering van sulfate en CSB.

Tweedens is mediese dripsakkies as anaerobiese bioreaktore gebruik om die mikrobiese diversiteit in SMW, wat met SDWWS behandel is, te bepaal deur verskeie veranderlikes te gebruik. Tweede generasie DNA-volgorde bepalingstegnieke is gebruik en data analises het gewys dat *Chlorobium* spp. die 90 d pionier toetslopie domineer met relatiewe persentasies van 68 % en 76 %. Transmissie elektron mikroskopie fotos en die helder groen kleur van die dripsakkies se vloeistof inhoud het die data analises bevestig. Die sulfate en CSB inhoud is onderskeidelik met > 98 % en > 85 % verminder.

‘n Korter behandelingstydperk is ondersoek met n 30 d pionier toetslopie. *Chlorobium* spp. was dominant, gevolg deur *Magnetospirillum* spp. en *Ornithobacterium* spp. Die vloeistof inhoud het na ‘n donker bruin kleur verander. Die CSB en sulfaat konsentrasies is met 60.8 % en 96 % onderskeidelik verminder na 26 dae waarna ‘n plato bereik is. Die effek van ‘n reeds bestaande biofilm in die bioreaktore het gewys dat *Chlorobium* spp. ook gedomineer het teen ‘n relatiewe persentasie van 62 % in vergelyking met die 36 % in die 30 d pionier toetslopie. ‘n Vermindering in sulfate en CSB van 96 % en 58 % is onderskeidelik is bereik binne 26 d

en die vloeistofinhoud was dieselfde kleur as die bioreaktore in die 30 d pionier toetslopie. Dit is moontlik dat die bruin Groen swawel bakterieë teenwoordig was. Daarom, ondanks ‘n groter teenwoordigheid van die *Chlorobium* spp. teen ‘n relatiewe persentasie in vergelying met die 30 d pionier toetslopie, was die verwydering van CSB en sulfate soortgelyk. Tydens die 30 d toetslopies het ‘n wit neerslag aan die bokant van die bioreaktore gevorm wat hoofsaaklik uit sulfaat en koolstof bestaan het wat ook ‘n aanduiding van die teenwoordigheid van *Chlorobium* spp. is. ‘n Toetslopie wat by laer temperature uitgevoer is kon die sulfate en CSB met slegs 10 % en 12 % onderskeidelik verminder nadat ‘n plato na 17 d bereik is. *Ornithobacterium* spp. het die eerste toetslopie gedomineer waar *Magnetospirillum* spp. die tweede toetslopie gedomineer het.

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Chapter 1

Introduction

Acid mine drainage is wastewater generated by mine and industrial activity (Geremias *et al.*, 2003). AMD is converted to a potential hazardous pollutant when exposed to oxygen, water and micro-organisms, as it results in the formation of sulphuric acid (Nordstrom & Alpers, 1999; Baker & Banfield, 2003; Johnson & Hallberg, 2003), posing a threat to the environment and human health alike. Exposure to untreated AMD may result in genotoxicity (Keller *et al.*, 2005) and a decrease in species diversity (Lee *et al.*, 2010).

AMD often does not adhere to the water quality guidelines of South Africa for domestic use (DWAF, 1996) and this has led to the investigation of possible treatment and remediation procedures with the aim of reducing the sulphate and heavy metal content, often associated with AMD (Nordstrom & Alpers, 1999).

Abiotic AMD treatment involve the addition of alkaline chemicals and materials but is often not economical (Skouen, 1991; Nairn *et al.*, 1992) whereas biotic treatments mainly involve the use of microbial bioreactors (Garcia *et al.*, 2001; Kappler & Dahl, 2001) and constructed wetlands (Colleran *et al.*, 1995; Khan *et al.*, 2009; Stottmeister *et al.*, 2013). However these methods require longer treatment periods when dealing with large volumes of wastewater (Pulles *et al.*, 2009).

Microbial treatment systems with regards to the treatment/remediation of AMD rely on the creation of anaerobic conditions for the reduction of sulphate. The micro-organisms involved commonly include sulphate reducing bacteria (Barton & Fauque, 2009). In order to create an anaerobic environment a suitable carbon source is required (Zdyb, 1999; Coetser *et al.*, 2000; Strosnider *et al.*, 2011a-c) but should be carefully considered as the carbon source has been shown to be the rate limiting step when using bioreactors for the treatment of AMD (Pulles *et al.*, 2003). Kikuyu grass, hay (Zdyb, 1999), sheep manure mixed with limestone and poultry manure mixed with limestone (Gilbert, 2004) have been identified as suitable carbon sources delivering sulphate reduction of up to 99 % when used as a carbon source in bioreactors during AMD treatment operations. The investigation of suitable carbon sources lead to the invention of the passive sulfate reducing treatment technology and the integrated and managed passive treatment (IMPI) process (Pulles & Heath, 2009). Despite the performance deliveries of the IMPI process the mentioned process still have drawbacks that need to be addressed including the time required for treatment (Pulles & Heath, 2009).

Domestic wastewater as a carbon source during the semi-passive treatment of AMD is successful in terms of sulphate, COD and heavy metal removal as well as the neutralization of the pH and it allows for the co-treatment of AMD and municipal wastewater (Davison *et al.*, 1989; Strosnider *et al.*, 2011b & 2011c; Sánchez-Andrea *et al.*, 2012; Hughes *et al.*, 2013). The co-treatment of AMD and domestic wastewater aims to bypass the active utilization of resources (Strosnider *et al.*, 2011b) and as domestic wastewater is readily available, it assists the responsibility of treating municipal wastewater sludge as third world countries often do not treat municipal wastewater sludge or have insufficient treatment operations (Gadgil, 1998; Strosnider *et al.*, 2011a). However, the composition of domestic wastewater sludge varies (Bhatti *et al.*, 1995; Tao *et al.*, 2012), making the investigation using domestic wastewater sludge difficult and the results unrepeatable.

In order to investigate the treatment of AMD with domestic wastewater sludge, a synthetic medium simulating domestic wastewater sludge was needed in order to study the treatment in a more universal way than currently available.

The aims of this study were to:

- Develop a synthetic medium simulating the BOD and COD of domestic wastewater sludge.
- Determine the ratio of AMD: synthetic domestic wastewater sludge (SDWWS) for optimal sulphate and COD removal.
- Investigate the influence of incubation temperature, treatment time and presence of a biofilm in bioreactors on the removal of sulphate and COD.
- Investigate the microbial population present in the respective trials by using next generation sequencing.

References

Baker, B. J., & Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, 44(2), 139-152.

Barton, L. L., & Fauque, G. D. (2009). Biochemistry, physiology and biotechnology of sulphate-reducing bacteria. *Advances in Aapplied Microbiology*, 68, 41-98.

Bhatti, Z. I., Furkukawa, K., & Fujita, M. (1995). Comparative composition and characteristics of methanogenic granular sludges treating industrial wastes under different conditions. *Journal of fermentation and bioengineering*, 79(3), 273-280.

Coetser, S.E., Cloete, T.E., & Zdyb, L. (2000). Biological sulphate reduction in artificial acid mine drainage using different carbon sources, Proceeding Y2K Millennium Meeting, Grahamstown 23-28 January, 606.

Colleran, E., Finnegan, S., & Lens, P. (1995). Anaerobic treatment of sulphate-containing waste streams. *Antonie van Leeuwenhoek*, 67(1), 29-46.

Davison, W., Reynolds, C. S., Tipping, E., & Needham, R. F. (1989). Reclamation of acid waters using sewage sludge. *Environmental Pollution*, 57(3), 251-274.

DWAF, Water quality guidelines for South Africa: First Edition 1996.

Gadgil, A. (1998). Drinking water in developing countries. *Annual review of energy and the environment*, 23(1), 253-286.

Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L., & Gonzalez, F. (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering*, 14(9), 997-1008.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Favere, V. T., Stolberg, J., Menezes, C. T. B., & Laranjeira, M. C. M. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental technology*, 24(12), 1509-1515.

Gilbert, O., De Pablo, J., Cortina, J.L., & Ayora, C. (2004). Chemical characterization of natural organic substrates for biological mitigation of acid mine drainage. *Water Research*, 38(19), 4186-4196.

Hughes, T. A., Gray, N. F., & Guillamón, O. S. (2013). Removal of metals and acidity from acid mine drainage using liquid and dried digested sewage sludge and cattle slurry. *Mine Water and the Environment*, 32(2), 108-120.

Johnson, D. B., & Hallberg, K. B. (2003). The microbiology of acidic mine waters. *Research in Microbiology*, 154(7), 466-473.

Kappler, U., & Dahl, C. (2001). Enzymology and molecular biology of prokaryotic sulfite oxidation1. *FEMS microbiology letters*, 203(1), 1-9.

Keller, J., Owens, C. T., Lai, J. C., & Devaud, L. L. (2005). The effects of 17 beta-estradiol and ethanol on zinc- or manganese-induced toxicity in SK–N–SH cells. *Neurochemistry International*, 46, 293–303.

Khan, S., Ahmad, I., Shah, M. T., Rehman, S., & Khaliq, A. (2009). Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management*, 90(11), 3451-3457.

Lampert, C., Zessner, M., & Kroiss, H. Sewage sludge composition – a multifunctional information. pp 1003-1009, unpublished date.

Lee, K. Y., Kim, K. W., & Kim, S. O. (2010). Geochemical and microbial effects on the mobilization of arsenic in mine tailing soils. *Environmental Geochemistry and Health*, 32, 31–44.

Nairn, R. W., Hedin, R. S., & Watzlaf, G.R. (1992). *Generation of alkalinity in an anoxic limestone drain*. Proc. of the 9th Annual National Meeting of the American Society for Surface Mining and Reclamation, Duluth, Minnesota.

Nordstrom, D. K., & Alpers, C. N. (1999). Geochemistry of acid mine waters. *The Environmental geochemistry of mineral deposits. part a: processes, techniques, and health issues*, 6, 133-160.

Pulles, W., Rose, P., Coetser, L., & Heath, R. (2003). Development of Integrated Passive Water Treatment Systems for the Treatment of Mine Waters, Proceedings of ICARD Conference, 12-18 July, Cairns, Australia.

Pulles, W., & Heath, R. (2009). The evolution of passive mine water treatment technology for sulphate removal, Golder Associates Africa (Pty) Ltd, PO Box 6001, Halfway House, 1685, South Africa. International Mine Water Conference.

Sánchez-Andrea, I., Triana, D., & Sanz, J. L. (2012). Bioremediation of acid mine drainage coupled with domestic wastewater treatment. *Water Science & Technology*, 66(11), 2425-2431.

Skousen, J., Lilly, R., & Hilton, T. (1991). Special chemicals for treating acid mine drainage. *Green Lands*, 34-41.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011a). Novel passive co-treatment of acid mine drainage and municipal wastewater. *Journal of environmental quality*, 40(1), 206-213.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011b). Alkalinity generation in a novel multi-stage high-strength acid mine drainage and municipal wastewater passive co-treatment system. *Mine Water and the Environment*, 30(1), 47-53.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011c). Biochemical oxygen demand and nutrient processing in a novel multi-stage raw municipal wastewater and acid mine drainage passive co-treatment system. *Water Research*, 45(3), 1079-1086.

Stottmeister, U., Wiebner, A., Kuschk, P., & Kappelmeyer, U. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances*, 22, 93–117.

Tao, J., Wu, S., Sun, L., Tan, X., Yu, S., & Zhang, Z. (2012). Composition of Waste Sludge from Municipal Wastewater Treatment Plant. *Procedia Environmental Sciences*, 12, 964-971.

Zdyb, L. (1999). Microbial sulfate reduction as a method of passive treatment of acid mine drainage using undefined carbon sources. University of Pretoria. South Africa, pp 67.

Chapter 2

Literature review

1. Introduction

Acid mine drainage is wastewater with a low pH, rich in pyrite, sulphur species and heavy metals generated by coal and gold mining (Geremias *et al.*, 2003). Through biological and chemical processes the pyrite is converted into acid mine drainage a hazardous pollutant when exposed to oxygen, water and micro-organisms (Nordstrom & Alpers, 1999b; Benner *et al.*, 2000; Johnson & Hallberg, 2003; Baker & Banfield, 2003).

Numerous micro-organisms take advantage of the heavy metal and sulphur rich, acidic niche created including iron oxidizers (Bond *et al.*, 2000), chemotrophs (Kishimoto *et al.*, 1991), members of the α- proteobacteria (Kusel *et al.*, 1999) and β-proteobacteria groups (Johnson *et al.*, 2001) to name but a few. In addition, posing competition to the sulfate reducers are the methanogens which dominate under low sulfate conditions (Winfrey & Zeikus, 1977; Maclnerney & Bryant, 1981).

According to the South African water quality guidelines, sulphate levels exceeding 200 mg/L is unsuitable for use for domestic purposes (DWAF, 1996). Wastewater generated by mining activities may contain sulphate levels as high as 1500 mg/L (Van der Merwe & Lea, 2003). Furthermore, exposure to untreated AMD may result in severe environmental (Lee *et al.*, 2010) and health issues (Keller *et al.*, 2005; Netto *et al.*, 2013) causing genotoxicity (Netto *et al.*, 2013) and provoking various medical disturbances in humans (Keller *et al.*, 2005), biota (Arun *et al.*, 2005; Tamás *et al.*, 2006) and aquatic systems (Peplow & Edmonds, 2005).

The treatment of AMD is therefore a necessity and has led to the research and development of various commercially available treatments methods. These methods include both biotic and abiotic methods of which alkaline treatment (Skousen, 1991) and passive systems such as constructed wetlands (Collins *et al.*, 2005; Khan *et al.*, 2009; Stottmeister *et al.*, 2003) or microbial bioreactors (Garcia *et al.*, 2001; Kappler & Dahl, 2001) are the preferred options.

All microbial treatment systems rely on creating anaerobic conditions for the reduction of sulphate. In order to create these conditions a suitable carbon source is required. Suitable carbon sources have been investigated in the quest to design the optimum sulfate reducing treatment operation (Zdyb, 1999; Coetser, *et al.*, 2000; Strosnider *et al.*, 2011a-c). Using such a suitable carbon source led to the invention of the passive sulfate reducing treatment

technology and the integrated and managed passive treatment (IMPI) process (Pulles & Heath, 2009). However, although these technologies deliver acceptable performance in terms of sulphate reduction, long periods of time are required which is not affordable for a high treatment demand (Pulles & Heath, 2009).

This review will focus on the generation of AMD, the microbial interaction involved and remediation methods used to improve the quality of AMD to comply with the standards of wastewater discharge.

2. Generation of AMD

In natural environments sulphur occurs predominantly as pyrite (Muyzer & Stams, 2008) which is associated with coal and gold deposits (McCarthy, 2011). Upon exposure to oxygenated water the pyrite is oxidized via biotic and abiotic reactions (A´lvarez *et al.*, 1993), producing metal sulphides and sulphuric acid (Costello, 2003). The acidic wastewater referred to as acid mine drainage (AMD) is then released into the environment, polluting aquatic systems (Delistraty & Yokel, 2007).

Research conducted in the early 21st century describes the oxidation of pyrite to yield sulphides and Fe^{3+} (Costello, 2003; Druschel *et al.*, 2004; Coetser & Cloete, 2005). The oxygenation of pyrite occurs in a two-step process. During the first phase, ferric iron and sulphuric acid are produced (equations 1 and 2).

$$FeS_2 + 2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2+} + 2H^+ \quad (1)$$

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + H_2O \quad (2)$$

In the second phase ferric hydroxide is precipitated, which is responsible for the characteristic orange colour of AMD.

$$Fe^{2+} + ¼O + 3H_2O \rightarrow Fe\ (OH)_3 + 3H^+ \quad (3)$$

At a lower pH ferric iron oxidizes pyrite completely, leading to the production of sulphuric acid (Sand *et al.*, 2001).

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2+} + 16H^+ \quad (4)$$

Other metal sulphides such as ZnS and PbS present may be partially oxidized by iron and protons via the polysulfide mechanism (Sand *et al.*, 1995) (equations 5 and 6).

$$8MeS + 8Fe^{3+} + 8H^{+} \rightarrow 8Me^{2+} + 4H_2S + 8Fe^{2+} \quad (5)$$

$$8H_2S + 8Fe^{3+} \rightarrow S_8^{o} + 8Fe^{2+} + 8H^{+} \quad (6)$$

However, sulphur-oxidizing bacteria are needed to complete the oxidation; equation 7.

$$S_8^{o}\ 12O_2 + 8\ H_2O \rightarrow 8SO_4^{-} + 16\ H^{+} \quad (7)$$

3. Microbial interactions in AMD

Nutrients are cycled via biogeochemical pathways, involving numerous micro-organisms. Examples of these biogeochemical pathways include the carbon, nitrogen and sulphur cycles. This study will focus on the sulphur cycle and the relevant organisms, due to the important role sulphur plays in the generation and toxicity of AMD.

3.1 The Sulphur cycle

The sulphur cycle is believed to be one of the earliest metabolic cycles of life on earth in which micro-organisms play an essential part. As previously mentioned, sulphur is also a main contributor to the production of AMD. This study will describe the influence of bacteria on the generation and remediation of AMD.

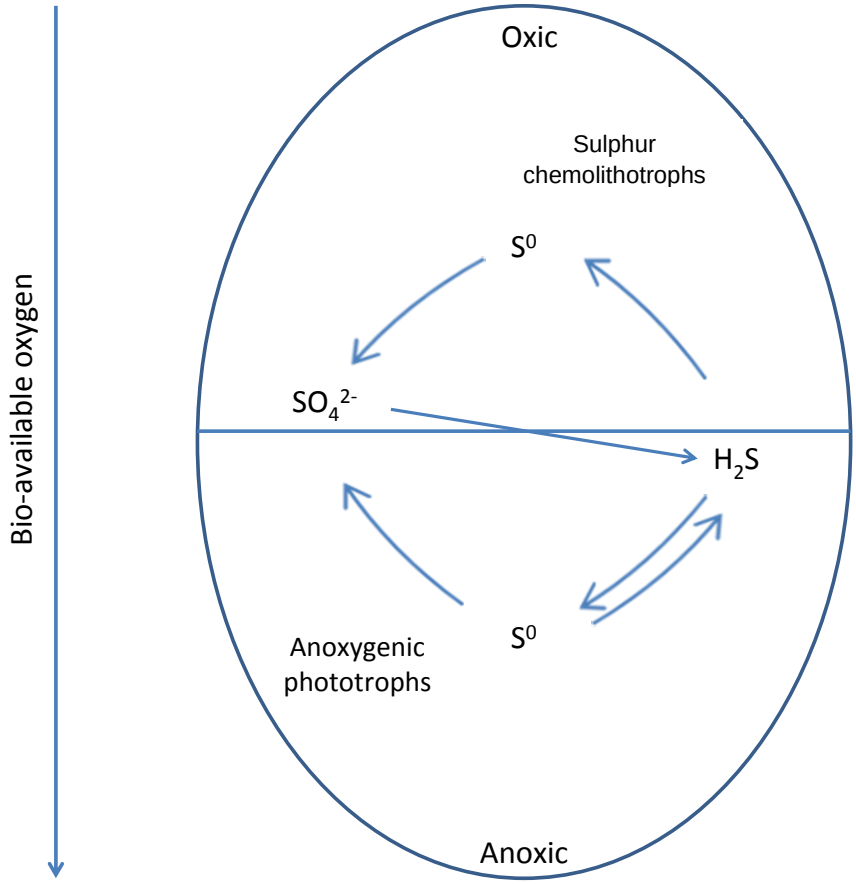

Figure 1. A schematic representation of the sulphur cycle in AMD (adapted from Brüser *et al.*, 2000; Madigan *et al.*, 2006).

The prokaryotes responsible for the aerobic conversion of elemental sulphur to sulphate and hydrogen sulfide to elemental sulphur include the chemolithotrophic bacteria and the cyanobacteria. Under anaerobic conditions green and purple sulphur bacteria oxidize elemental sulphur to sulphate (Brune, 1995; Frigaard & Dahl, 2008). Sulphate reducing bacteria reduce elemental sulphur to hydrogen sulfide via dissimilative sulphate reduction (Barton & Fauque, 2009).

3.2 Microbial oxidation of sulphur

In the natural environment the sulphur related metabolic activities of the chemolitotrophs and photolithotrophs together form the oxidative half of the sulphur cycle; under aerobic conditions sulphur is oxidized to sulphate whereas sulphate is reduced to sulphur under anaerobic conditions (Brüser *et al.*, 2000) (Figure 1). However, products of the sulphur metabolic pathways lead to the generation of AMD (Nordstrom & Alpers, 1999a,b). Chemolithotrophs refers to micro-organisms using the aerobic oxidation of inorganic compounds and CO_2 fixation via various biochemical pathways in order to obtain cellular

energy (Thauer, 2007). Bacterial oxidation of sulphur species may occur under basic (Sorokin *et al.,* 2001), neutral or acidic (Harrison, 1984) conditions in the presence of oxygen. The bacteria include the genera *Acidithiobacillus* (Kelly & Wood, 2000), *Beggiatoa* (Strohl, 1989), *Pseudomonas* (Friedrich & Mitrenga, 1981; Kuenen & Beudeker, 1982), *Sulfolobus* (Stetter *et al.,* 1990), *Thermithiobacillus* (Kelly & Wood, 2000), *Thiobacillus* (Kuenen & Beudeker, 1982; Kelly *et al.,* 2000), *Xanthobacter*, *Thiomicrospira* and *Thiosphaera* (Kuenen *et al.,* 1982) species responsible for the biotic production of AMD.

$$2S^{\circ} + 4\,O_2 \rightarrow 2\,SO_4^{2-} \qquad (8)$$

$$2\,S^{\circ} + 3\,O_2 + 8\,H_2O \rightarrow 2\,H_2SO_4^{2-} \qquad (9)$$

$$O_3^{2-} + O_2 + 3H_2O \rightarrow 2SO_4^{2-} + 6H^{+} \qquad (10)$$

The S4-intermediate pathway whereby thiosulphate is oxidized by *Acidithiobacillus ferrooxidans* has been studied (Masau *et al.,* 2001; Beard *et al.,* 2011). The first reaction involves the thiosulphate dehydrogenase (TD) catalyzed oxidation of two thiosulphate molecules whereby four tetrathionate molecules are formed. The second step is the hydrolysis of tetrathionate by the enzyme tetrathionate hydrolase (TTH) whereby sulphate and disulfane monosulfonic acid are produced (Steudel *et al.,* 1987). Sulphate, thiosulphate, and elemental sulphur are produced due to the high reactivity nature of TTH (De Jong *et al.,* 1997). Tetrathionate hydrolase in *Acidithiobacillus* spp. have been identified (Kanao *et al.,* 2007) and characterized (De Jong *et al.,* 1997).

3.3 Iron oxidation by Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans not only obtains cellular energy by oxidizing elemental sulphur but also via the oxidation of ferric iron using dissolved oxygen as a final electron acceptor (Rohwerder *et al.,* 2003b). The ability of iron oxidizers to oxidize iron (II) ions increases its contribution to the generation of AMD (Johnson & Hallberg, 2003). *A. ferrooxidans* tolerates metals at much higher concentrations than other micro-organisms (Hutchins *et al.,* 1986; Dopson *et al.,* 2003) and is a popular choice for bioleaching (Rohwerder *et al.,* 2003b).

Schippers and colleagues (Schippers *et al.,* 1996; Schippers & Sand, 1999) indicated that different pathways are used for the oxidation of different metal sulphides. Schippers & Sand (1999) proposed the following reaction in the case of pyrite oxidation through the thiosulphate mechanism (equations 11 and 12). Oxidation via the thiosulphate mechanism

occurs through a ferric iron attack on metal sulphides with sulphate being the main end product.

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow S_2O_3^{2-} + 7\ Fe^{2+} + 6\ H^+ \quad (11)$$

$$S_2O_3^{2-} + 8\ Fe^{2+} + 3\ H_2O \rightarrow SO_4^{2-} + 8\ Fe^{2+} + 6\ H^+ \quad (12)$$

An enzymatic pathway, namely the SOX-system, thought to be responsible in neutrophilic bacteria, is absent in Gram-negative proteobacteria (Müller *et al.*, 2004). However, another enzyme, known as sulphur diooxygenase (SDO) is thought to be responsible for sulphur oxidation in Gram-negative proteobacteria as SDO activity was demonstrated by Rohwerder and Sand (Rohwerder & Sand, 2003a). Unfortunately SDO in proteobacteria has not been characterized (Rohwerder & Sand 2003a; 2007).

$$1/8\ S8 + O_2 + H_2O \rightarrow SO_3^{2-} + 2\ H^+ \quad (13)$$

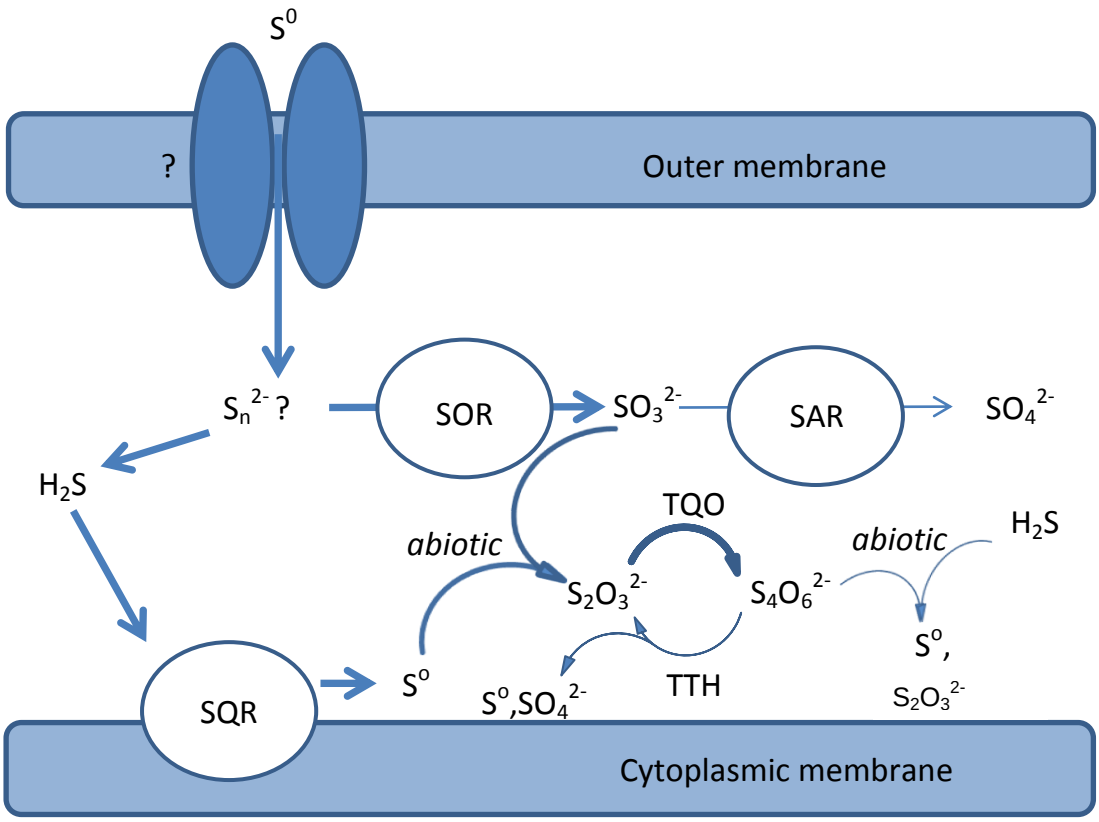

Figure 2. A schematic representation of inorganic sulphur oxidation by proteobacteria (adapted from Rohwerder & Sand 2003a; 2007). The main reactions are indicated in bold arrows.

Sulphur diooxygenase is located in the periplasmic space (Figure 2). Elemental sulphur (S°) has to cross the outer membrane and enter the periplasmic space in order for sulphur oxidation to take place. As shown by Rohwerder and Sand (Rohwerder & Sand, 2003a), SDO only oxidizes highly active thiol-bound sulfane sulphur atoms (R-SSnH) and not S° or sulfide, therefore thiol containing proteins (R-SH) in the outer membrane and periplasmic space serve as a sulphur transport mechanism. Sulfite is produced in the diooxygenase reaction which is oxidized to sulphate. However, the mechanism in *At. ferrooxidans* is much more intricate as enzymes catalyzing sulfide oxidation (SQR) and thiosulphate (TQO) are active (Brasseur *et al.*, 2004; Wakai *et al.*, 2004). SQR and TQO activity can be explained by the formation of sulfide as a side reaction via the reaction of free thiol groups of proteins involved in the transport mechanism with sulfane sulphur-containing groups. Thereafter the oxidation of sulfide to elemental sulphur follows which can be transferred and oxidized by SDO, or alternatively undergoes chemical oxidation to thiosulphate. TQO oxidizes the thiosulphate further to tetrathionate (Rohwerder & Sand, 2007).

3.4 Sulphur oxidizing archaebacteria

Archaea from the orders *Euryarchaeota* and *Crenarchaeota* use elemental sulphur as final electron acceptor (Liu *et al.*, 2012). Certain archaea belonging to the order *Sulfolobales* grow mixotrophically and can utilize iron as a final electron acceptor (Karavaiko *et al.*, 2006; Schippers, 2007). Certain species within the *Crenarchaeota* have unique gene combinations allowing the aerobic oxidation of sulphur and anaerobic sulphate reduction (Segerer *et al.*, 1985; Zillig *et al.*, 1985).

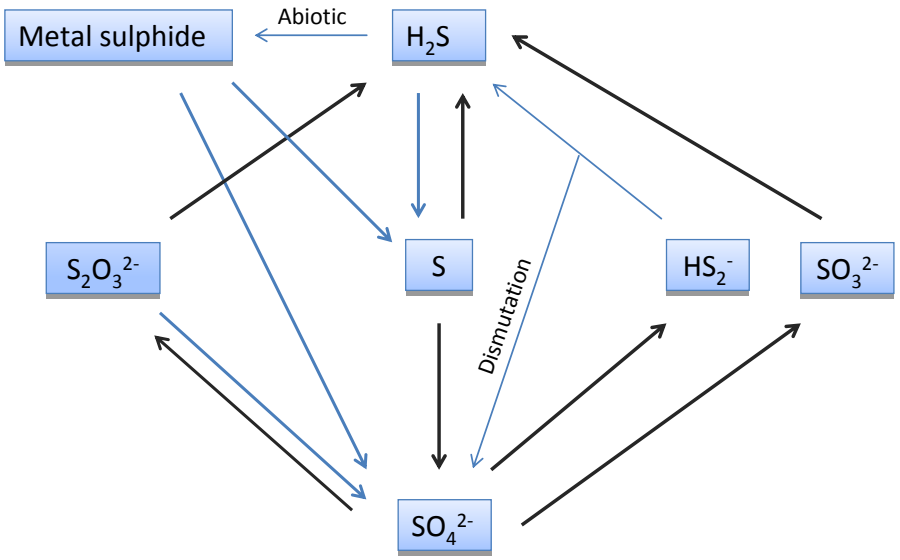

Figure 3. A schematic representation of the sulphur cycle (adapted from Offre *et al.*, 2013).

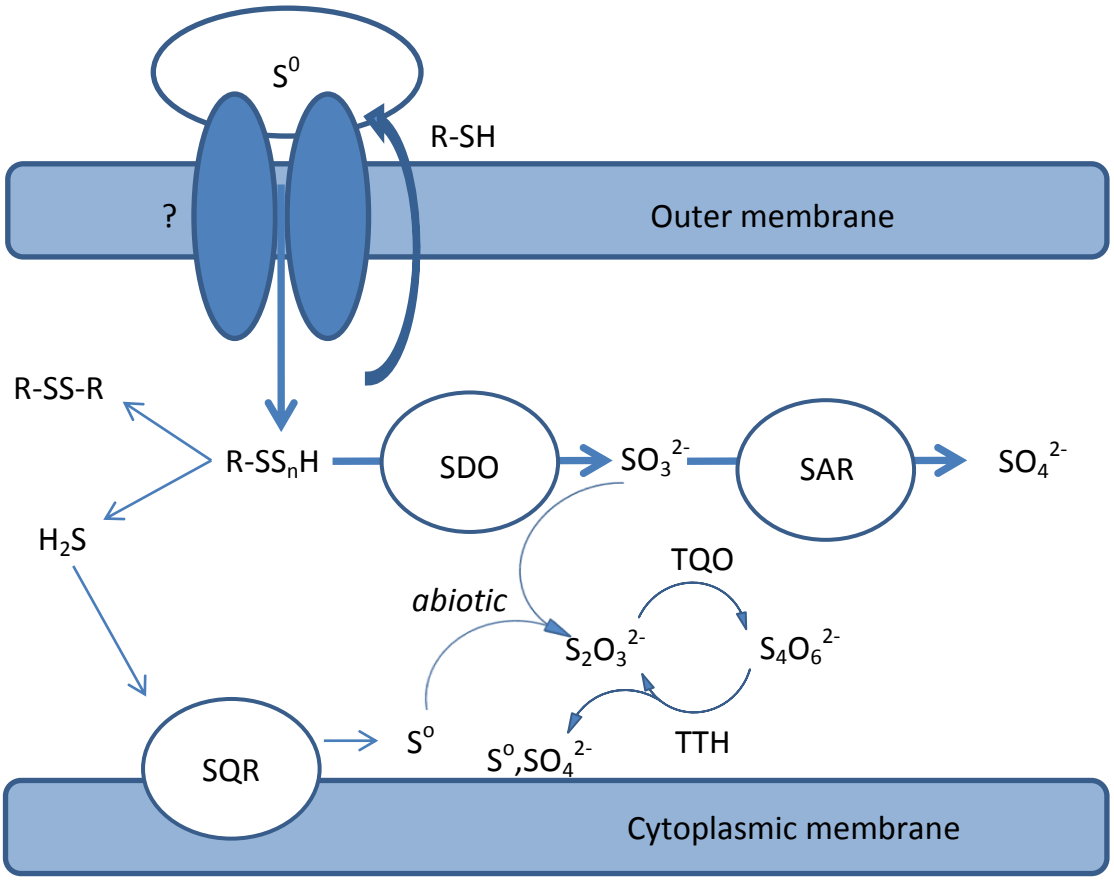

Figure 4. A proposed model for the archael oxidation of elemental sulphur. The main routes are indicated in bold arrows (adapted from Rohwerder and Sand (Rohwerder & Sand, 2003a).

3.5 Phototrophs

Phototrophs consist of phototrophic purple bacteria such as the *Allochromatium* spp. (Pfennig & Trüper, 1992), purple non-sulphur bacteria *(Rhodospirillaceae*), green sulphur oxidizing bacteria (Overmann & Tuschak, 1997; Imhoff, 2008) and green non sulphur bacteria known as cyanobacteria.

3.5.1 Purple and green non-sulphur bacteria

Purple non-sulphur bacteria (PNS) refers to an extremely versatile, non-taxonomic group of facultative anoxygenic prokaryotes able to use sulphur as a final electron acceptor, although not to the same degree as purple and green sulphur bacteria (Brock *et al.*, 2003). PNS belongs to the class *Alphaproteobacteria* and includes the orders *Rhodospirillales*, *Rhodobacterales* and *Rhizobiales*.

PNS can grow photoautotrophically utilizing either hydrogen as electron donor and reducing CO_2 via the CBB–Benson–Bassham (CBB) cycle, use fixed carbon compounds as both carbon source and electron donor or grow chemoautotrophically, reducing CO_2 (Tabita, 1988; 1999). *Rhodopseudomonas palustris* is a popular choice for microbial biodegradation of chemical wastes and complex carbon compounds (Sasikala & Ramana, 1998; Egland *et al.*, 2001) due to their versatile metabolism.

Green non-sulphur bacteria is the collective name given to non-sulphur chlorophilic prokaryotes which covers a range of different metabolic and phylogenetic types including aerobic strains (Chang *et al.*, 2011; Löffler *et al.*, 2013), photoautotrophs (e.g. *Chloroflexus aurantiacus*) (Tang *et al.*, 2011) and fermentative *Anaerolinea thermophila* (Yamada *et al.*, 2006). Filamentous *Chloroflexus* spp. have been detected in AMD impacted regions (Senko *et al.*, 2008) and may inhabit microbial mats in geothermal springs with a neutral alkalinity using different metabolic pathways in order to generate cellular energy (Klatt *et al.*, 2013; Zarzycki *et al.*, 2009). Green non-sulphur bacteria are also present in abundance in activated sludge wastewater treatment systems (Seviour & Blackall, 1999).

3.5.2 Purple and green sulphur oxidizing bacteria

During the oxidation of sulfide to sulphur both GSB and PSB form sulphur globules as intermediates (Pott & Dahl, 1998; Frigaard & Dahl, 2008). PSB fully oxidize sulfide to sulphate (Pott & Dahl, 1998) which is deposited as sulphur chains (Prange, 2002) in globules in the periplasm (Pattaragulwanit, 1998) encapsulated by a protein (Brune, 1995).

Green sulphur oxidizing bacteria (GSB) are obligate anaerobes belonging to the family *Chlorobiaceae* (Overmann & Tuschak, 1997; Imhoff, 2008) that occur in dimly lit anoxic environments (Van Gemerden & Mas, 1995). Cellular energy is generated through anoxygenic photosynthesis (Pfenning, 1989), a process whereby solar energy is converted to ATP without producing oxygen with reduced sulphur compounds such as sulfide, thiosulphate and elemental sulphur as electron donors (Dahl & Prange, 2006; Goh *et al.*, 2009). A few strains have been identified to be able to oxidize Fe^{2+} (Heising *et al.*, 1999). Anoxygenic photosynthesis occurs via the light-harvesting centrums (chlorosomes packed with antenna bacteriochlorophylls) situated on the inside of the cell membranes (Blankenship *et al.*, 1995) (Figure 5). Various metabolic pathways and groups of enzymes are used by different sulphur oxidizing bacteria (SOB) such as the sulphur-oxidizing (SOX), sulfide quinone oxidoreductase (SQR) and the dissimilatory sulfite reductase (Dsr) enzyme systems (Friedrich *et al.*, 2001; Sander *et al.*, 2006; Chan *et al.*, 2009).

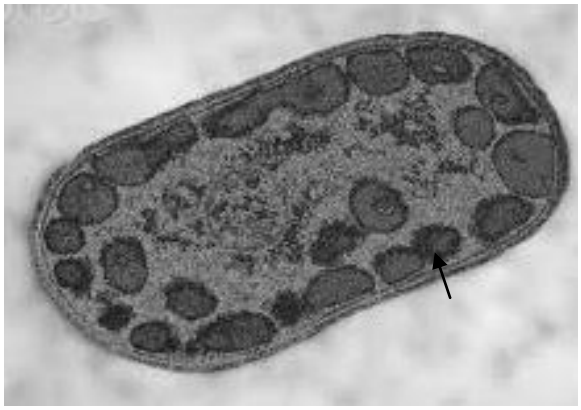

Figure 5. Electron microscope photo of a *Chlorobium* cell by Dr. Terry Beveridge/Visuals Unlimited/Corbis ©. Visible on the inside of the membrane are the chlorosomes.

Chlorobium tepidum is the model organism of green sulphur bacteria due to its complete genomic sequence being documented (Eisen *et al.*, 2002) and natural transformability (the ability of *C. tepidum* to incorporate suitable external DNA) (Frigaard & Bryant, 2001). Other members of the pylum include *Chlorobium thiosulfatophilum*, *Chlorobium phaeobacteroides* and *Chlorobium limicola*.

Due to their robustness and the nature of their sulphur metabolism, anoxygenic sulphur bacteria have been widely used in wastewater treatment processes worldwide as they enable a cost effective, low maintenance solution to sulfide rich organic wastewater (Malik *et al.*, 2008; Mara, 2008; Moura *et al.*, 2009; Office of National Assessment, 2009).

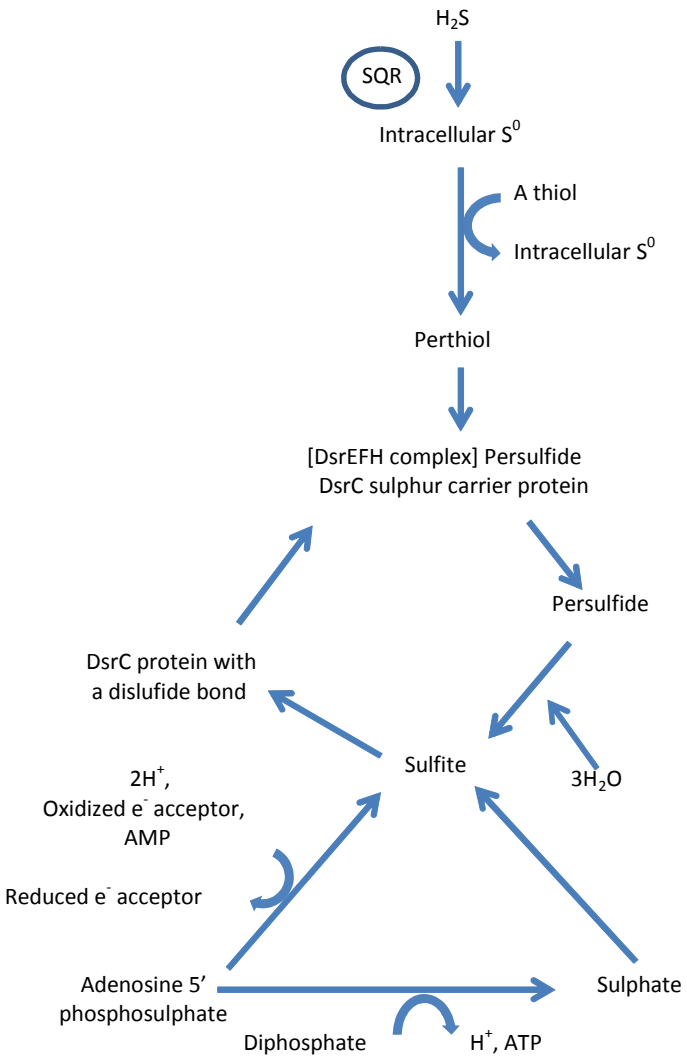

Figure 6. A schematic representation of the super sulphur reducing pathway in photolithotrophic bacteria (Caspi *et al.*, 2010).

The initial sulfide oxidation begins with the transfer of an electron from a sulfide molecule to the quinine pool, mediated by a periplasmic sulfide:quinone oxidoreductase (Figure 6). According to Pott and Dahl (Pott & Dahl, 1998), sulphur globules are produced which are further reduced to HS^- or perthiols and then further oxidized to sulfide, catalyzed by dissimilatory sulfite reductase (Dsr). Disulfide formation in the DsrC follows the transfer of the perthiol to the Dsr. DsrK of the Dsr renders the disulfide active again (Loy, 2009; Grein *et al.*, 2010).

3.6 Microbial reduction of sulphur

Microbial reduction of sulphate plays an essential part of the sulphur cycle. Sulphate reducing bacteria (SRB) is the main bacterial group involved in sulphate reduction and occupies a vast variety of anaerobic niches including anaerobic biofilms (Devereux *et al.*, 1992), activated sludge (Manz *et al.*, 1998) and aquatic sediments (Sahm *et al.*, 1999). SRB can essentially be

divided into four groups when considering their use of sulphate as a final electron acceptor during anaerobic respiration (Table 2), namely Gram-negative mesophilic SRB (e.g. Deltaproteobacteria), Gram-positive spore forming SRB from the Firmicutes division, thermophilic bacterial SRB and thermophilic archaeal SRB from the genera Archaeoglobus and crenarchaeotal (Widdel & Bak, 1992; Castro *et al.*, 2000; Rabus *et al.*, 2006; Muyzer & Stams, 2008; Barton & Fauque, 2009).

SRB utilize inorganic sulphate as a terminal electron acceptor obtained through the oxidation of organic substrates and the reduction of sulphate or molecular hydrogen to hydrogen sulfide (LeGall & Fauque, 1988) (equation 11), a process known as dissimulatory sulphate reduction. This characteristic leads to SRB induced bio-corrosion (Bermont-Bouis *et al.*, 2007) and biotechnological applications (see review Hockin & Gadd, 2007), the harvesting of electricity (Lee *et al.*, 2012) and heavy metal bioremediation (Zhou *et al.*, 2013).

ATP activates cytoplasmic sulphate, a reaction catalyzed by sulphate adenylyltransferase, resulting in the generation of pyrophosphate and adenosine 5'-phosphosulphate (APS) (Figure 7). Thereafter adenylylsulphate reductase catalyses the reduction of APS to sulfite and AMP. The electron donor for this reaction has yet to be identified. The reduction of sulphite to sulphide is catalysed by Dsr, the main energy conserving step in sulphate respiration. In contrast to sulphate oxidation in phototrophic bacteria as previously discussed, the Dsr is responsible for sulphur reduction.

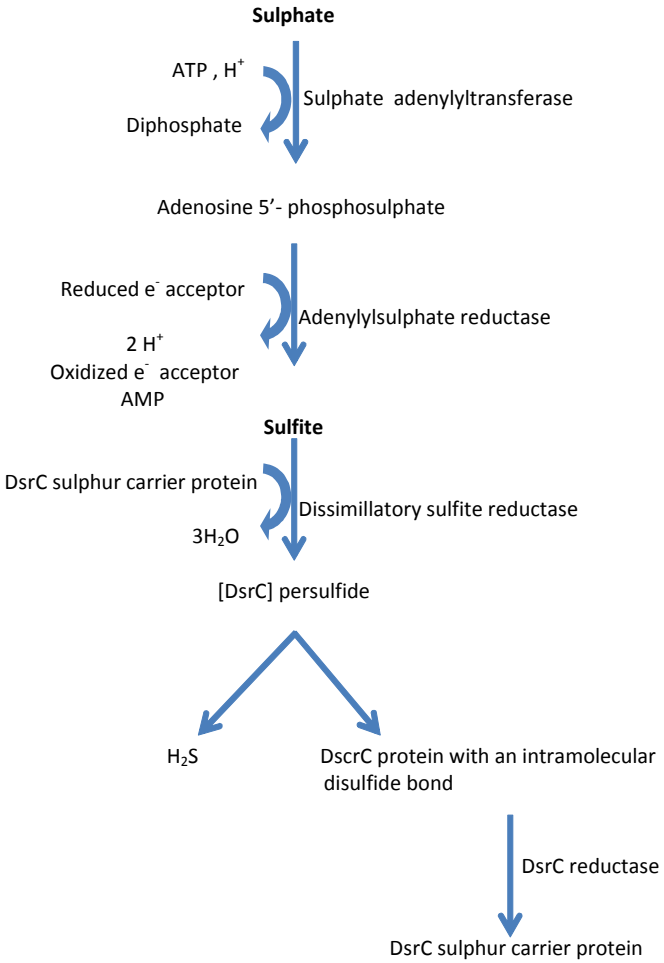

Figure 7. Sulphate dissimilation by SRB. (Created 27 May 1999 by Pellegrini-Toole A, Marine Biological Laboratory Revised 21-Sep-2006 by Caspi R , SRI International Revised 10-Dec-2010 by Caspi R , SRI International). The enzymes involved are indicated on the right hand side of the respective arrows.

Although the process of sulfite reduction has yet to be determined, a mechanism has been proposed by Oliveira *et al.* (Oliveira *et al.*, 2008) and Grein *et al.* (Grein *et al.*, 2010). The proposed mechanism involves the DsrC sulphur carrier protein being presulphurated by DsrAB at a conserved cysteine residue and then dissociates. The persulfide is reduced at the second cysteine residue. Hydrogen sulfide is released and an intramolecular disulfide in DsrC is formed. It is possible that the DsrK subunit of the DsrMKJOP transmembrane complex catalyses the regeneration of the DsrC.

$$SO_4^{2-} + CH_3COOH + 2\ H^+ \rightarrow HS^- + 2\ HCO_3^- + 3\ H^+ \quad (11)$$

Due to SRB's ability to utilize various substrates as energy sources (Rabus *et al.*, 2006) they have diverse metabolic types and may be heterotrophic, autotrophic or litho autotrophic (Cypionka, 1995) and is a popular choice in AMD remediation treatments which will be discussed.

3.7 Methanogens vs. sulfate reducers: competition and coexistence

Methanogens are not part of the sulphur cycle but do play a significant role in the alterations of AMD. Not only has their presence in AMD in great numbers been indicated (Buccambuso *et al.*, 2007) competing with SRB for nutrients (Isa *et al.*, 1986; Yoda *et al.*, 1987; Choi & Rim, 1991) and sulfates (Colleran *et al.*, 1995).

Methanogens play in important role in the oxidation of substrate to carbon dioxide and/or methane under anaerobic conditions (Winfrey & Zeikus, 1977; Maclnerney & Bryant, 1981). Under non-limiting sulfate conditions SRB outcompete methanogens for substrates including acetate, hydrogen and formate (Takacs *et al.*, 2001) which can be explained based on their kinetic properties (Ward & Winfrey, 1985; Widdel, 1988). However, under low sulfate conditions methanogens will dominate SRB (Stams, 1994). Methanogens detected in acidic environments include *Methanosarcinales, Methanobacteriales* and *Methanomicrobiales* (Steinberg & Regan, 2008).

4. Remediation of AMD

The characteristics and possible remediation treatments for AMD have been investigated from the mid 1900's (Olem & Unz, 1977; Wieder, 1989). Remediation can involve abiotic or biological treatment methods each divided into active and passive systems (Johnson & Hallberg, 2005). The aim of such passive treatment systems is to reduce sulfate and organic matter content and to neutralize the pH of the AMD. The focus of this study is passive anaerobic treatment for sulphate reduction in AMD and will therefore be discussed in more detail.

4.1 Active abiotic remediation of acid mine drainage

Active treatment implies the need for a constant addition of an active key ingredient (most often a suitable carbon source) to the treatment process. The downside of this approach is that the sludge precipitating upon pH alterations (Dempsey & Jeon, 2001) requires additional steps to process the sludge before it can be disposed of.

Active abiotic treatments include the addition of neutralising agents such as sodium hydroxide and limestone (Nairn *et al.*, 1992).

In the presence of water, Calcium oxide (CaO) forms calcium hydroxide ($Ca(OH)_2$). The chemical reaction upon the addition of quicklime can be described as follows:

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (14)$$

$$Ca(OH)_2 \rightarrow Ca^{2+} + 2OH^- \quad (15)$$

As the pH increases (equation 14), metal ion precipitation precipitates as hydroxides (equation 15). The rate of precipitation is dependent on various environmental factors such as pH, temperature and metal concentrations.

$$Mg^{2+} + 2OH^- \rightarrow Mg(OH)_2 \quad (16)$$

4.2 Passive abiotic remediation of acid mine drainage

Passive abiotic treatment implies the absence of active addition of chemicals or neutralising agents and the system depends solely on gravitational forces natural geochemical processes.

Anoxic limestone drains (ALD) serves as an example. AMD flows through limestone filled trenches via gravity. As the anoxic mine wastewater makes contact with the limestone, alkalinity is added to the wastewater (Skousen, 1991). ALD systems are cost effective and maintenance is simple relative to alternative treatments (Skousen, 1991).

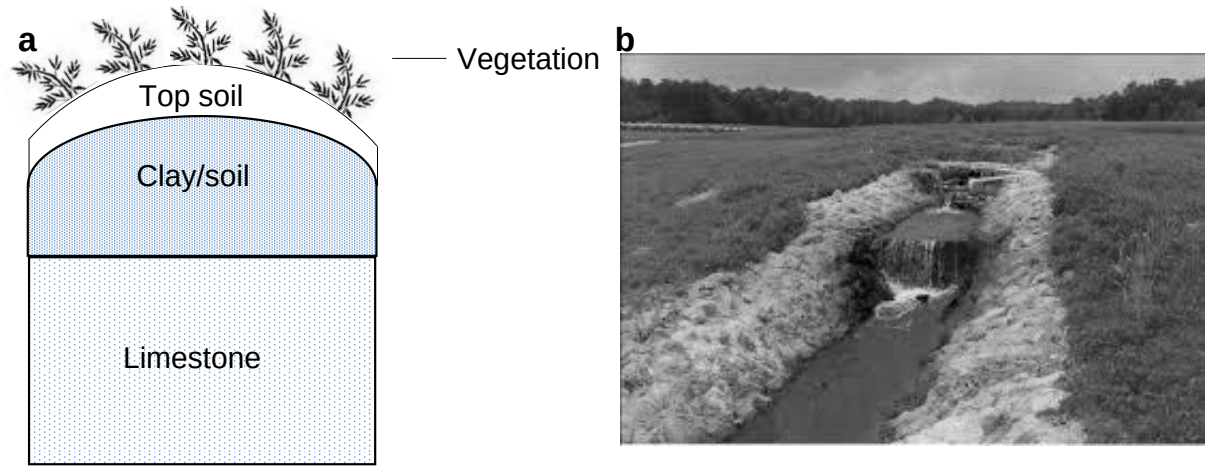

Figure 8. a) A schematic representation of the design of an anoxic limestone drain (adapted from Interstate Technology and Regulatory Council (2010)). b) An ALD system at Midwestern reclamation site, Pike County, Indiana. Photo by Denver Harper. The vegetation surrounding the ALD indicates successful remediation of the acid mine drainage.

4.3 Active biotic remediation of acid mine drainage

With the rising demand for environmentally friendly treatments the use of microbial based methods has become popular including for example wetlands and bioreactors.

Phytoremediation as applied in wetlands is the process whereby hydrophilic plants and their associated micro-organisms are used to remove water contaminants such as heavy metals and solvents via various metabolic pathways. Constructed wetlands are manmade wetlands which make use of phytoremediation in order to restore the pH, heavy metal concentrations (Collins *et al.*, 2005; Khan *et al.*, 2009; Stottmeister *et al.*, 2003) and toxins (Allende *et al.*, 2011) of wastewater to levels suitable for irrigation in a natural, passive, non-chemical fashion.

The use of wetlands for the treatment of mine wastewater was first explored in the 1990's (Mine Environment Neutral Drainage Program 1990; 1993). When constructed directly on mine tailings impoundments it may reduce the formation of AMD (Stoltz, 2003). Wetlands can be categorised according to function and configuration as eithersurface flow, subsurface flow or vertical flow systems (Mthembu *et al.*, 2013). Substrates including plants and sand are used (Johnson & Hallberg, 2005) to create environments required for optimal remediation of AMD of a specific nature.

Aerobic wetlands aid in the remediation of AMD by allowing heavy metals to be removed in an environment simulating an aerobic natural environment.

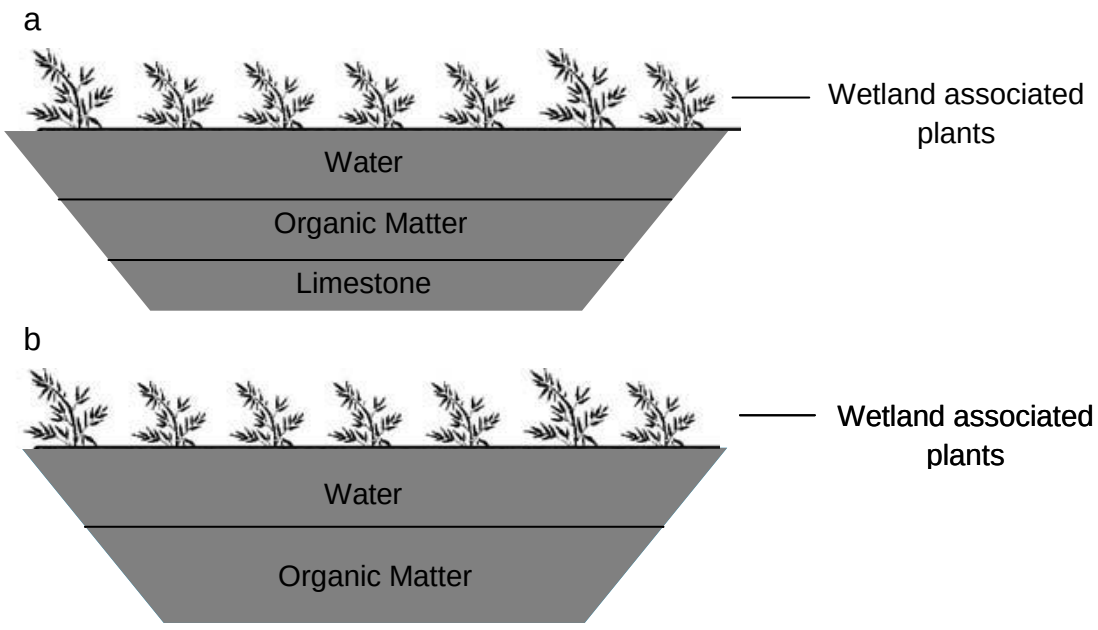

Figure 9. A schematic representation of a) an anaerobic constructed wetland and b) an aerobic constructed wetland (Skousen, 1991).

Anaerobic wetlands remediate heavy metals and sulphate rich AMD by providing conditions under which metals are precipitated (Costello, 2003). Biological substrates are incorporated to allow metabolic reduction of sulphates by micro-organisms such as sulphate reducing bacteria (SRB), neutralisation of acidity and the precipitation of metals.

4.4 Passive biotic remediation of acid mine drainage

The objective of passive remediation treatment systems is reduction of the sulphates present in AMD by providing a niche for sulphate reducing bacteria (SRB) (Garcia *et al.*, 2001; Kappler & Dahl, 2001; Bijmans *et al.*, 2010; Burns *et al.*, 2012; Sánchez-Andrea *et al.*, 2012). Although the role SRB play in the remediation of AMD has been well studied, insight into the bacterial community involved in sulfate reducing technology is needed (Johnson & Hallberg, 2005; Pruden *et al.*, 2006), especially the identification of the micro-organisms contributing to the efficacy of these technologies.

A typical passive sulphate reducing bioreactor consists out of basin in which a substrate, a microbial inoculum and an alkaline agent are mixed. AMD enters the bioreactor via an inflow and undergoes treatment as it seeps through the bioreactor filled with organic matter. At the bottom of the basin a drainage systems allows the treated water to escape where after it is often released into a wetland (Figure 10).

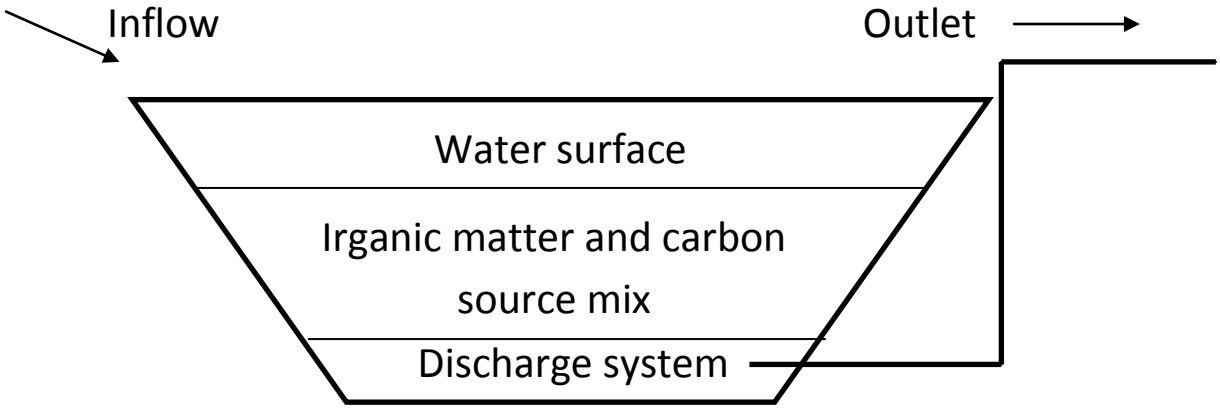

Figure 10. A schematic representation of a typical AMD sulfate reduction operation bioreactor (adapted from Gusek (2002)).

Zhang *et al.* (2013) showed that the addition of Fe (III) to sulphate rich wastewater was beneficial in treating the sulphate and chemical oxygen demand (COD) content of the wastewater. This novel treatment involves the addition of Fe_2SO_3 in a two-stage manner to two sequentially-connected reactors, the first being an acidogenic reactor and the second an acidogenic sulphate-reducing reactor. The first reactor resulted in microbial reduction of

sulphate and COD content by 57.9 % and 27.3 % respectively, compared to the 29 % and 15.6 % respectively obtained by the reference reactor. When combined, the sequential reactors delivered a COD reduction of 74.2 % (Zhang *et al.*, 2013).

The low concentration of bio available carbon present in AMD (Kolmert & Johnson, 2007) is the limiting factor for biological remediation. The properties of the carbon source are determining factors in the success of a sulphate reducing treatment (White & Gadd, 1999; Béchard *et al.*, 1994) as it determines the rate-limiting step (Pulles *et al.*, 2003). A carbon source which supports sulphate reduction for a prolonged period without the need for nutritional supplementation is preferred. Various carbon sources have been investigated for the use of SRB bioreactors including used manure and sawdust matrices, cheese whey (Drury, 1999; Coetser *et al.*, 2000) lactate (El Bayoumy *et al.*, 1999), ethanol (Nagpal *et al.*, 2000), whey (Christensen *et al.*, 1996), amino acids and hydrogen (White, 1995). Different options for bioreactor matrices have also been studied including rock, wood and plastic (Steed *et al.*, 2000).

Zdyb (1999) investigated the efficacy of domestic sewage sludge, molasses, and cow manure as well as various grasses as carbon sources for SRB bioreactors for the remediation of AMD. Anaerobic digester sludge was used as inoculum. It was found that kikuyu grass and digester sludge were the best performing carbon sources obtaining sulphate reduction rates of 138.25 mg/L/d and 128.5 mg/L/d respectively. The good performance of the kikuyu grass may be attributed to the ability of the lactic acid inhabitants present to digest sugars and cellulose present in the grass cuttings, making simpler carbon sources available to the SRB (Fenton, 1987). Dill and co-workers (Dill *et al.*, 2001) showed sulphate reduction of 97.8 % and 99 % with kikuyu grass and hay respectively as carbon sources in AMD treatment. Gilbert *et al.* (Gilbert *et al.*, 2004) combined lime stone with carbon sources to reduce sulphates in AMD and found 80 % sulphate reduction in the combination of oak leaf with lime stone and 99 % reduction in the combinations of both sheep manure and poultry manure with limestone.

Chang *et al.* (2000) demonstrated the advantage biologically or chemically treated carbon sources have above untreated substrates for the treatment of AMD and the significance of nutritional supplementation and SRB inoculation during the early stages of sulphate reducing AMD treatment.

Pulles and Heath (2009) investigated the evolution of passive mine water treatment technology with reference to the removal of sulphate, sulphur oxidation and bio-neutralization

over a period of fourteen years in order to design a successful passive treatment technology. This led to the design of numerous passive AMD treatment technologies.

According to Pulles and Heath (2009) a successful passive sulphate reduction treatment needs to be coupled with a passive sulfide oxidation technology to remove sulphides resulting from sulphate reduction before they can be re-oxidized to sulphate. The typical performance of a passive sulphate reducing treatment reactor can be divided into five basic phases (Pulles & Heath, 2009). During the initial phase the bacterial populations of the reactor adapt to the environment, hence the sulphate reduction rate is low. This lag phase may last up to 90 days.

The sulphate reduction during the second phase is highly effective but does not typically last longer than eight months after the start-up of the reactor. The high efficiency is dedicated to an abundance of readily available energy in the hydrolysable lignocellulose for sulphate reduction. The third phase is defined by a sharp decline in sulphate reduction as the readily hydrolysable lignocellulose has been exhausted after eight to nine months of reactor start-up. With the absence of lignocellulose supplementation the reduction of sulphate will be reduced but at a stable rate for a period of five to six years. Eventually sulphate reduction will cease due to an inability to hydrolyse the remaining lignocellulose.

Pulles and Heath (2009) also described the integrated and managed passive treatment process. When it came to light that the lignocellulose hydrolysis rate determines the performance of the sulphate reduction reactors ways to overcome this obstacle were researched. This led to the initiation of what was named the integrated and managed passive treatment (IMPI) process.

The main aim of the IMPI process is to unlock the energy contained within the lignocellulose for the reduction of sulphate at rates high enough to produce a technology economically viable. The research followed two approaches; the pre-treatment of lignocellulose to increase the rate at which anaerobic bacteria can utilize it and secondly, to study the mechanisms of anaerobic lignocellulose hydrolysis in order to develop more effective optimizations for this rate limiting step.

The IMPI process consists of four stages. The first reactor (the degrading packed bed reactor (DPBR)) is essentially a reactor consisting out of multiple layers of selected carbon sources and is supplemented with these carbon sources on a regular basis. The purpose of the DPBR is the rapid conditioning of the influent by establishing desired redox conditions and to produce sufficient sulphides and alkalinity, as well as to optimize the hydrolysis of lignocellulose and the production of volatile fatty acids, thus providing an effluent rich in

sulphides, volatile fatty acids, nutrients and an optimum pH and low levels of metals and sulphate.

The second reactor is known as the primary sulfide oxidizing bioreactor (PSOB) of which the purpose is to oxidize sulphides to elemental sulphur to be removed from the system, preventing the oxidation of sulphides back to sulphates. Carbon sources are absent with minimal changes to the remainder of the effluent of the DPBR.

A specific selection of carbon sources is present in the reactor following the PSOB, the secondary sulphate reducing reactor (SSRR). In this reactor the volatile fatty acids are utilized and additional sulphates are removed in order to obtain desired sulphate levels (known as the design level, established as 300 millimoles per cubic metre per day (mM/m3/d) (Gusek, 1998). The effluent produced by the SSRR contains the components of the effluent produced by the DPBR at reduced levels.

The purpose of the final reactor, the secondary sulfide oxidizing bioreactor (SSOB) is the oxidation of sulphides to elemental sulphur in order to be removed from the system. A final aerobic step may be applied to remove residual volatile fatty acids and nutrients if needed.

The individual reactors may be combined in a custom designed fashion, for example one DPBR to various SSRR units, depending on the purpose of the reactors.

When treating AMD a common problem is the decreased efficiency of technologies such as the DPBR when treating wastewater of a pH of 4.5 or less. In order to overcome this problem Pulles and Heath (2009) proposed a design for a technology able to passively increase the alkalinity to treat water with a pH as low as 2.8 and water rich in heavy metals using microbial bioreactors consisting out of three main microbial populations.

The purpose of the first population is the removal of oxygen from the system. The main species involved are facultative anaerobes. The second population involves the degradation of lignocellulose to simple carbon compounds by various micro-organisms, e.g. *Clostridium* spp. The final population is comprised out of SRB that are able to utilize the simple carbon compounds as electron donors for the reduction of sulphate to sulfide accompanied by the production of bicarbonates, resulting in an increase in alkalinity.

4.5 The use of municipal wastewater and sewage-sludge as carbon source in the treatment of AMD.

The use of domestic wastewater as a carbon source during the semi-passive treatment of AMD for the removal of sulphate and heavy metals has been investigated as it allows the co-treatment of acid mine drainage and municipal wastewater (Davison *et al.,* 1989; Strosnider *et al.,* 2011b-c; Hughes *et. al.,* 2013; Strosnider *et al.,* 2013).

Domestic wastewater treatment plants are known to have a significant environmental impact due to the large area the operation structures requires (Wilderer *et al.,* 2000) and the insufficient treatment of domestic wastewater in developing countries (Gadgil, 1998; Strosnider *et al.,* 2011a). Conventional domestic wastewater treatments require active input of resources for the additional removal of pathogens and solids (Metcalf & Eddy Inc., 2002). The co-treatment of AMD and domestic wastewater aims to bypass the active utilization of resources (Strosnider *et al.,* 2011b).

The use of domestic wastewater sludge as a carbon source in the passive treatment of AMD showed promising results in terms of AMD neutralization and metal absorption (Davison *et al.,* 1989; Hughes *et al.,* 2013). A few example studies will be discussed.

In 1989, Davison and co-workers investigated the use of sewage waste to neutralize the pH of an exhausted sand quarry (Davison *et al.,* 1989). The quarry was treated with calcium hydroxide to restore a neutral pH where after sewage was added to prevent any further production of acid. This state was maintained for a period of two years where after acidic conditions returned. This was due to the lake being too shallow and only half of the acid mine drainage contaminated area was covered. The addition of sewage sludge encouraged sulphate reduction however the basin had an unpleasant odour and shortly after initiation the organisms began to populate the quarry including phytoplankton and macrophytes (Davison *et al.,* 1989).

Sánchez-Andrea and co-workers (2012) used domestic wastewater by feeding anaerobic bioreactors with 1:10 (v:v) of a synthetic AMD: domestic wastewater mixture. Sediments from the Tinto River (Huelva, Spain) were used as inoculum. Half of the organic matter present in the domestic wastewater co-precipitated with metals present in the AMD, therefore the mixture had to be supplemented with acetate as an additional carbon source to achieve higher sulphur elimination. More than 88 % of COD and 75 % of the sulphate was removed, 85 % Fe and 99 % of other metals dissolved. The dominant bacterial species found in the bioreactors included two metabolic groups, namely fermentative and sulphate reducing bacteria of which *Clostridium* spp., *Delftia* spp., *Paludibacter* spp. and *Pelotomaculum* spp. (fermentative) and *Desulfomonile* spp., *Desulfovibrio* spp., *Desulfosporosinus* spp. and

Desulfotomaculum spp. (sulphate reducing) were the most abundant. It was also interesting to note the larger biodiversity present in the bioreactors compared to the biodiversity of the inoculum (Sánchez-Andrea *et al.*, 2012).

Strosnider and co-workers have investigated the co-treatment of acid mine drainage and municipal waste (Strosnider *et al.*, 2011b-c; Strosnider *et al.*, 2013). The system used by Strosnider (Strosnider *et al.*, 2013) comprised of four serial unit processes:

The initial processes were anoxic of nature and allowed for solids settling after the initial mixing of the municipal wastewater and acid mine drainage as well as microbial Fe(III) reduction and an increase in pH. The second and third unit processes emulated vertical flow bioreactors in anaerobic columns. Inert bio media in the upper column sections allowed for further dissolved oxygen stripping whereas limestone in the bottom parts of the columns encouraged abiotic alkalinity production. The forth unit processes consisted of aerobic wetland mesocosms for metal oxidation and precipitation. A reduction in nitrate and acidity was noted as well as the removal of a few metals such as Mn and Zn. The transformation of iron in solid phases was indicated by an increase in dissolved iron from 45.3 to 147 mg/l. This implied that the accumulated iron can potentially be remobilized and removed from the unit process (Strosnider *et al.*, 2013).

The removal of metals and neutralization of pH of synthetic AMD by digester sludge, cattle slurry or Biofert granules (dried granular anaerobic sludge) were compared (Hughes *et al.*, 2013). The influence of contact time and the concentration of the solids were also investigated. The removal of metals varied between the respective materials and the total metal concentration removed was directly proportional to the contact time. Copper, lead and aluminium were first to be removed followed by zinc and manganese. Overall the most metals were removed by cattle slurry. In terms of pH neutralization there was little difference between the cattle slurry and digester sludge. Neutralization of pH was reached within 30 min after the material was added to the synthetic AMD. The pH reached a maximum of 5.5 after addition of cattle slurry. In contrast, the Biofert granules neutralized the pH after 300 min and the pH remained at a maximum of 4.0 with the only exception at high solid concentrations (Hughes *et al.*, 2013).

Deng and Lin (2013) co-treated AMD and municipal wastewater by using a two-stage process involving the mixing of the two prior to an anaerobic biological treatment process. Various ratios of AMD to municipal wastewater showed a COD/sulphate concentration ratio of 0.05-5.4, a phosphate removal of 9-100 % and pH of 6.2-7.9. The treatment removed COD and

sulphate consistently by more than 80% from the mixtures for COD/sulphate ratios of 0.6-5.4. This study indicated promising results for the co-treatment of AMD and municipal wastewater using two-stage anaerobic treatment processes (Deng & Lin, 2013).

5. Identification of microorganisms by next generation sequencing

When studying microbial life, the study of the DNA component is virtually inevitable as it provides insight into various aspects of the microbial community ranging from the identification of the microbial species present to the evolutionary relations between microbial species (Chun & Rainey, 2014).

The first commercially applied method of DNA sequencing was the method developed by Sanger and co-workers in the 1970's known as the Sanger method (Sanger *et al.*, 1977). This method utilizes a catalytic reaction to synthesize DNA fragments complementary to the template DNA (DNA of interest).

Firstly, heat is applied to denature the double strand template DNA. A short 32P labelled oligonucleotide complementary to the template DNA, serving as a primer, is annealed to a specific known region on the template DNA which serves as a starting point for the DNA synthesis reaction. This reaction is conducted in four separate tubes, each containing a mixture of the four deoxynucleoside triphosphates (dNTPs) as well as an appropriate amount of a modified dNTP (also referred to as a terminator or ddNTP), a single type of ddNTP per tube. Thereafter DNA polymerase catalyses the polymerizations of deoxynucleoside triphosphates (dNTPs) onto the complementary DNA strain until a modified nucleoside is incorporated (Sanger *et al.*, 1977).

The resulting fragments contain the same 5' end, whereas the 3' end is determined by the ddNTP used in the reaction. After DNA synthesis in all four tubes has been completed all the DNA fragments are loaded onto denaturing polyacrylamide gel, each in their own parallel lane. The pattern of the bands is read by autoradiography (Sanger *et al.*, 1977).

However, the Sanger method is not without disadvantages. Firstly, this method is biologically bias as the DNA of interest is cloned into vectors that have to be compatible with the replication system of Escherichia coli cells which makes some parts of the genome practically unable to be cloned (Men *et al.*, 2008). Furthermore, a great limitation of the Sanger method is the inability to analyse allele frequencies (Men *et al.*, 2008). Lastly, the Sanger method is not economical (Men *et al.*, 2008).

The modern wave of sequencing technologies that followed the Sanger method and to a certain extend replaced it, is referred to as next generation sequencing (NGS) (The European Bioinformatics Institute).

NGS technologies are more economical viable, have a higher throughput and are significantly more accurate (The European Bioinformatics Institute) when compared to the Sanger sequencing method (Liu *et al*., 2012). NGS is an encompassing term used to describe the modern sequencing techniques available including Illumina (Solexa) sequencing, Roche 454 sequencing and Ion torrent: Proton/PGM/SOLiD sequencing. These will be briefly discussed shortly.

5.1 Illumina sequencing

When using the Illumina sequencing method the input DNA sample is cut into short fractions and annealed to a slide by means of adaptors (Mardis, 2008; The European Bioinformatics Institute). PCR is carried out where after the resulting products are flooded with DNA polymerase and one type fluorescent labelled terminator ddNTPs and an image of the slide is taken. In principal there will be one fluorescent ddNTP at each spot. The terminators are removed and the terminator of the next nucleotide is added until all the terminators were used. The sequence reads are determined by means of a computer. The length of the sequences obtained is determined by the amount of cycles undergone and all sequences are of the same length.

5.2 Roche 454 sequencing

In contrast to the Illumina sequencing method the input DNA samples is cut into short fractions of up to 1 kb which allows longer reads when compared to the 100-150 bp reads of the Illumina sequencing method (Mardis, 2008) The European Bioinformatics Institute; Roche Applied Science) The fragments undergo PCR using specific primers after being annealed to beads (each fragment onto a respective bead). Each bead is placed into a well of a slide along with DNA polymerase, sequencing buffers and one type of nucleoside triphosphate (NTP). When a nucleotide is added a light signal is released and is detected by the machine. The NTPs are removed and the cycle is repeated until all the nucleotides had an opportunity. The sequences obtained differ in length in contrast to the sequences obtained by the Illumina sequencing method.

5.3 Ion torrent: Proton/PGM/SOLiD sequencing

In contrast to the previously mentioned NGS sequencing methods the Ion torrent sequencing method does not use light signals but instead uses the phenomenon of the release of a H+ ion upon the addition of a dNTP to the DNA strand undergoing polymerases (Flusberg *et al.*, 2010; The European Bioinformatics Institute). Once again the input DNA is cut into short ~200bp fragments which are annealed onto beads (each fragment onto a respective bead). The fragments undergo PCR and the beads are placed into wells on a slide. As in the case of 454 sequencing the four types of dNTPs are added respectively along with sequencing buffers and DNA polymerase. The pH of the respective wells are monitored to detect the release of each H+ ion as the addition of H+ ion will lower the pH.

5.4 Applications of NGS

The ability to determine the presence of microbial genera and species is a powerful tool as it allows the study of specific external stimuli and/or other microbial organisms on the inhabiting microbial communities.

GATC biotechnology is the technology used to determine the bacterial composition present in food (The European Bioinformatics Institute). Wahl *et al.* (unpublished date) used NGS to characterize the bacterial composition of food. They identified the inhabiting bacteria in two brands of yogurt using NGS technologies and demonstrated the NGS technology's high level of sensitivity with regards to detection limit as it was able to detect 103 *E. coli* cells in 0.2 mL of yogurt. Daly and co-workers (2000) developed oligonucleotide probes and primers for the detection of phylogentic subgroups of sulphate reducing bacteria (SRB) in landfill leachate. PCR primers for the 16S rRNA gene of six of the phylogentic subgroups of SRB were designed and used these primers in conjunction with group-specific oligonucleotide probes in order to detect SRB. The results included the differentiation of five generic groups. The primers and oligonucleotide probes developed can be used to evaluate landfill site performance as the inhabiting SRB community may serve as a bio marker.

6. Conclusion

Acid mine drainage poses a threat to public and environmental wellbeing. Biotic treatments have been gaining ground over outdated chemical treatments as the use of wetlands and especially SRB have shown to be more effective. Using inexpensive carbon sources such as domestic waste may have additional benefits as it allows for more cost effective treatment methods. However, a clear understanding into the microbial communities present as well as the identification of the micro-organisms responsible for treatment is needed. Regarding the

microbial analyses of treatment systems it is important to keep in mind the method of analyses best suitable for the specific treatment option as various methods of microbial DNA analyses are available. Also, effective modern passive treatment systems including IMPI technologies and two-stage anaerobic treatment processes require long periods of time to be operational, a luxury the exposed environment cannot afford.

7. References

Allende, K. L., Fletcher, T. D., & Sun, G. (2011). Enhancing the removal of arsenic, boron and heavy metals in subsurface flow constructed wetlands using different supporting media. *Water Science and Technology*, 63(11), 2612-2618.

A´lvarez, E., Perez, A., & Calvo, R. (1993). Aluminium speciation in surface waters and soil solutions in areas of sulphide mineralization in Galicia (NW Spain). *Science of the total environment*, 133(1), 17-37.

Arun, K. S., Carlos, C., Herminia, L., & Avudainayagam, S. (2005). Chromium toxicity in plants, *Environmental International,* 31, 739–753.

Baker, B. J., & Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, 44(2), 139-152.

Benner, S.G., Gould, W.D., & Blowes, D.W. (2000). Microbial populations associated with the generation and treatment of acid mine drainage, *Chemical Geology*, 169, 435-448.

Barbério, A., Voltolini, J. C. & Mello, M. L. (2011). Standardization of bulb and root sample sizes for the Allium cepa test. *Ecotoxicology,* 20, 927–935.

Barton, L. L., & Fauque, G. D. (2009). Biochemistry, physiology and biotechnology of sulfate‐reducing bacteria. *Advances in Applied microbiology*, 68, 41-98.

Beard, S., Paradela, A., Albar, J.P. & Jerez, C.A. (2011). Growth of *Acidithiobacillus ferrooxidans* ATCC 23270 in thiosulphate under oxygen-limiting conditions generates extracellular sulphur globules by means of a secreted tetrathionate hydrolase. *Frontiers in Microbiology, Microbial Physiology and Metabolism* (2), Original research article.

Béchard, G., Yamazaki, H., Gould, W. D., & Bédard, P. (1994). Use of cellulosic substrates for the microbial treatment of acid mine drainage. *Journal of Environmental Quality*, 23(1), 111-116.

Bermont-Bouis, D., Janvier, M., Grimont, P. A., Dupont, I., & Vallaeys, T. (2007). Both sulfate-reducing bacteria and Enterobacteriaceae take part in marine bio-corrosion of carbon steel. *Journal of Applied Microbiology*, 102(1), 161-168.

Bijmans, M. F. M., De Vries, E., Yang, C., Buisman, C.J., Lens, P. N. L., & Dopson, M. (2010). Sulfate reduction at pH 4.0 for treatment of process and wastewaters. *Biotechnology Progress*, 26, 1029–1037.

Blankenship, R. E., Olson, J. M., & Miller, M. (1995). Antenna complexes from green photosynthetic bacteria. In *Anoxygenic photosynthetic bacteria* (pp. 399-435). Springer Netherlands.

Bond, P.L., Smriga, S.P., & Banfield, J.F. (2000). Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Applied Environment Microbiology* ,66, 3842-3849.

Brasseur, G., Levican, G., Bonnefoy, V., Holmes, D., Jedlicki, E., & Lemesle-Meunier, D. (2004). Apparent redundancy of electron transfer pathways via bc1 complexes and terminal oxidases in the extremophilic chemolithoautotrophic *Acidithiobacillus ferrooxidans*. *Biochimica et Biophysica Acta*, 1656, 114–126.

Brune, D. C. (1995). Isolation and characterization of sulphur globule proteins from *Chromatium vinosum* and *Thiocapsa roseopersicina*. *Archives of Microbiology*, 163(6), 391-9.

Bruser, T., Lens, P., & Truper, H. (2000). The biological sulfur cycle. *Environmental technologies to treat sulfur pollution-Principles and Engineering, Lens, PNL and Hulshoff PoLL. Ed, International Water Association, London*, 47-85.

Buccambuso, E., Figueroa, L., Ranville, J., Wildeman, T., & Reisman, D. (2007). Microbial activity in the Peerless Jenny King sulfate reducing bioreactor system. *Proclamation of Amererican Society of Mining and Reclamation, Gillette, WY*, 109-122.

Burns, A. S., Pugh, C. W., Segid, Y. T., Behum, P. T., Lefticariu, L., & Bender, K. S. (2012). Performance and microbial community dynamics of a sulfate-reducing bioreactor treating coal generated acid mine drainage. *Biodegradation,* 2(3), 415-29.

Caspi, R., Foerster, H., Fulcher, C. A., Kaipa, P., Krummenacker, M., Latendresse, M., Karp, P. D. (2008). The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic acids research, 36* (suppl 1), D623-D631.

Castro, H.F., Williams, N.H., & Ogram, A. (2000). Phylogeny of sulfate-reducing bacteria. *FEMS Microbiology Ecology,* 31, 1–9.

Chan, L. K., Morgan-Kiss, R. M., & Hanson, T. E. (2009). Functional analysis of three sulfide: quinone oxidoreductase homologs in *Chlorobaculum tepidum*. *Journal of Bacteriology,* 191, 1026–1034.

Chang, Y.J., Land, M., Hauser, L., Chertkov, O., Del Rio, T.G., Nolan, M., Copeland, A., Tice, H., Cheng, J.F., Lucas, S., Han, C., Goodwin, L., Pitluck, S., Ivanova, N., Ovchinikova, G., Pati, A., Chen, A., Palaniappan, K., Mavromatis, K., Liolios, K., Brettin, T., Fiebig, A., Rohde, M., Abt, B., Göker, M., Detter, J.C., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., et al: (2011). Non-contiguous finished genome sequence and contextual data of the filamentous soil bacterium *Ktedonobacter racemifer* type strain (SOSP1-21). *Stand Genomic Science,* 5, 97–111.

Chun, J., & Rainey, F. A. (2014). Integrating genomics into the taxonomy and systematics of the Bacteria and Archaea. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 2), 316-324.

Choi, E., & Rim, J. M. (1991). Competition and inhibition of sulfate reducers and methane producers in anaerobic treatment. *Water Science & Technology*, 23(7-9), 1259-1264.

Christensen, B., Laake, M., & Lien, T. (1996). Treatment of acid mind water by sulphate-reducing bacteria: results from a bench scale experiment. *Water Resources*, 30, 1617–1624.

Coetser, S.E., Cloete, T.E., & Zdyb, L. (2000). Biological sulphate reduction in artificial acid mine drainage using different carbon sources, Proceeding Y2K Millennium Meeting, Grahamstown 23-28 January, 606.

Coetser, S.E., & Cloete, T.E. (2005). Biofouling and biocorrosion in industrial water systems. *Critical reviews in microbiology*, 31(4), 213-232.

Collins, B.S., Sharitz, R.R., & Coughlin, D.P. (2005). Elemental composition of native wetland plants in constructed mesocosm treatment wetlands. *Bioresource technology*, 96(8), 937-948.

Colleran, E., Finnegan, S., & Lens, P. (1995). Anaerobic treatment of sulphate-containing waste streams. *Antonie van Leeuwenhoek*, 67(1), 29-46.

Costello, C. (2003). Acid Mine drainage: Innovative Treatment technologies, National Network of Environmental Management Studies, viewed 18 June 2013, from http://www.cluin.org/download/studentpapers/costello_amd.pdf.

Cypionka, H. (1995). Solute transport and cell energetics. In *Sulphate-reducing bacteria* (pp. 151-184). Springer US.

Dahl, C., & Prange, A. (2006). Bacterial sulfur globules: occurrence, structure and metabolism. In *Inclusions in prokaryotes* (pp. 21-51). Springer Berlin Heidelberg.

Davison, W., Reynolds, C. S., Tipping, E., & Needham, R. F. (1989). Reclamation of acid waters using sewage sludge. *Environmental Pollution*, 57(3), 251-274.

De Jong, G. A., Hazeu, W., Bos, P., & Kuenen, J. G. (1997). Polythionate degradation by tetrathionate hydrolase of Thiobacillus ferrooxidans. *Microbiology*, 143(2), 499-504.

Delistraty, D., & Yokel, J. (2007). Chemical and ecotoxicological characterization of Columbia River sediments below the Hanford site (USA). *Ecotoxicology and environmental safety*, 66(1), 16-28.

Dempsey, B. A., & Jeon, B. H. (2001). Characteristics of sludge produced from passive treatment of mine drainage. *Geochemistry: Exploration, Environment, Analysis*, 1(1), 89-94.

Deng, D., & Lin, L.S. (2013). Two-stage combined treatment of acid mine drainage and municipal wastewater. *Water Science & Technology*, 67(5), 1000-1007.

Devereux, R., Kane, M.D., Winfrey, J., & Stahl, D.A. (1992). Genus-and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *Systematic and applied microbiology*, 15(4), 601-609.

Dill, S., Cloete, T.E., Coetser, L., & Zdyb, L. (2001). Determination of the suitability of alternative carbon sources for sulphate reduction in the passive treatment of mine water. *WRC Report:* 802/1/01.

Dopson, M., Baker-Austin, C., Koppineedi, P.R., & Bond, P.L. (2003). Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms. *Microbiology*, 149(8), 1959-1970.

Drury, W. J. (1999). Treatment of acid mine drainage with anaerobic solid-substrate reactors. *Water Environment Research*, 1244-1250.

Druschel, G. K., Baker, B. J., Gihring, T. M., & Banfield, J. F. (2004). Acid mine drainage biogeochemistry at Iron Mountain, California. *Geochemical Transactions*, 5(2), 13-32.

DWAF, Water quality guidelines for South Africa: First Edition 1996.

Egland, P. G., Gibson, J., & Harwood, C. S. (2001). Reductive, Coenzyme A-Mediated Pathway for 3-Chlorobenzoate Degradation in the Phototrophic Bacterium Rhodopseudomonas palustris. *Applied and environmental microbiology*, 67(3), 1396-1399.

Eisen, J. A., Nelson, K.E., Paulsen, I.T., Heidelberg, J.F., Wu, M., Dodson, R.J., Deboy, R., Gwinn, M.L., Nelson, W.C., Haft, D.H., Hickey, E.K., Peterson, J.D., Durkin, A.S., Kolonay, J.L., Yang, F., Holt, I., Umayam, L.A., Mason, T., Brenner, M., Shea, T.P., Parksey, D., Nierman, W.C., Feldblyum, T.V., Hansen, C.L., Craven, M.B., Radune, D., Vamathevan, J., Khouri, H., White, O., Gruber, T.M., Ketchum, K.A., Venter J.C., Tettelin, H., Bryant, D.A., & Fraser, C.M. (2002). The complete genome sequence of Chlorobium tepidum TLS, a photosynthetic, anaerobic green-sulphur bacterium. *Proceedings of the National Academy of Science USA, 99***,** 9509-9514.

El Bayoumy, M.A., Bewtra, J.K., Ali, H.I., & Biswas, N. (1999). Sulfide production by sulfate reducing bacteria with lactate as feed in an upflow anaerobic fixed film reactor. *Water Air Soil Pollution*, 112, 67–84.

Fenton, M. P. (1987). An investigation into the sources of lactic acid bacteria in grass silage. *Journal of applied bacteriology*, 62(3), 181-188.

Friedrich, C. G., & Mitrenga, G. (1981). Oxidation of thiosulfate by Paracoccus denitrificans and other hydrogen bacteria. *FEMS Microbiology Letters*, 10(2), 209-212.

Frigaard, N. U., & Bryant, D. A. (2001). Chromosomal gene inactivation in the green sulfur bacterium Chlorobium tepidum by natural transformation. *Applied and environmental microbiology*, 67(6), 2538-2544.

Frigaard, N. U., & Dahl, C. (2008). Sulfur metabolism in phototrophic sulfur bacteria. *Advances in microbial physiology*, 54, 103-200.

Friedrich, C. G., Rother, D., Bardischewsky, F., Quentmeier, A., & Fischer, J. (2001). Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism?. *Applied and Environmental Microbiology*, 67(7), 2873-2882.

Gadgil, A. (1998). Drinking water in developing countries. *Annual review of energy and the environment*, 23(1), 253-286.

Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L., & Gonzalez, F. (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering*, 14(9), 997-1008.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Favere, V. T., Stolberg, J., Menezes, C. T. B., & Laranjeira, M. C. M. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental technology*, 24(12), 1509-1515.

Gilbert, O., De Pablo, J., Cortina, J.L., & Ayora, C. (2004) Chemical characterization of natural organic substrates for biological mitigation of acid mine drainage. *Water Research*, 38(19), 4186-4196.

Goh, S. H. M., Mabbett, A. N., Welch, J. P., Hall, S. J., & McEwan, A. G. (2009). Molecular ecology of a facultative swine waste lagoon. *Letters in applied microbiology*, 48(4), 486-492.

Grein, F., Pereira, I. A., & Dahl, C. (2010). Biochemical characterization of individual components of the Allochromatium vinosum DsrMKJOP transmembrane complex aids understanding of complex function in vivo. *Journal of bacteriology*, 192(24), 6369-6377.

Gusek, J. J., Wildeman, T.R., Miller, A. and Fricke, J. (1998). "The Challenges of Designing, Permitting and Building a 1,200-GPM Passive Bioreactor for Metal Mine Drainage, West Fork Mine, Missouri," in Proceedings of the 15th Annual Meeting, ASSMR, St. Louis, Missouri, May 17-21.

Gusek, J. J. (2002). Sulfate-reducing bioreactor design and operating issues: Is this the passive treatment technology for your mine drainage. *National Association of Abandoned Mine Land Programs, Park City, Utah*.

Harrison Jr, A. P. (1984). The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. *Annual Reviews in Microbiology*, 38(1), 265-292.

Heising, S., Richter, L., Ludwig, W., & Schink, B. (1999). Chlorobium ferrooxidans sp. nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "Geospirillum" sp. strain. *Archives of microbiology*, 172(2), 116-124.

Herlihy, A. T., Kaufmann, P. R., Mitch, M. E., & Brown, D. D. (1990). Regional estimates of acid mine drainage impact on streams in the Mid-Atlantic and Southeastern United States. *Water, Air, and Soil Pollution*, 50(1-2), 91-107.

Hockin, S. L., Gadd, G. M., Barton, L. L., & Hamilton, W. A. (2007). Bioremediation of metals and metalloids by precipitation and cellular binding. *Sulphate-reducing Bacteria: Environmental and Engineered Systems*.

Hughes, T. A., Gray, N. F., & Guillamón, O. S. (2013). Removal of metals and acidity from acid mine drainage using liquid and dried digested sewage sludge and cattle slurry. *Mine Water and the Environment*, 32(2), 108-120.

Hutchins, S. R., Davidson, M. S., Brierley, J. A., & Brierley, C. L. (1986). Microorganisms in reclamation of metals. *Annual Reviews in Microbiology*, 40(1), 311-336.

Imhoff, J. F. (2008). Systematics of anoxygenic phototrophic bacteria. In *Sulfur Metabolism in Phototrophic Organisms* (pp. 269-287). Springer Netherlands.

Isa, Z., Grusenmeyer, S., & Verstraete, W. (1986). Sulfate reduction relative to methane production in high-rate anaerobic digestion: technical aspects. *Applied and Environmental Microbiology*, 51(3), 572-579.

Interstate Technology and Regulatory Council, 2010, *Anoxic Limestone Drains.* Washington, D.C.: Interstate Technology and Regulatory Council, Mining Waste Team.

Johnson, D. B., Rolfe, S., Hallberg, K. B., & Iversen, E. (2001). Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. *Environmental Microbiology*, 3(10), 630-637.

Johnson, D. B., & Hallberg, K. B. (2003). The microbiology of acidic mine waters. *Research in Microbiology*, 154(7), 466-473.

Johnson, D. B., & Hallberg, K. B. (2005). Acid mine drainage remediation options: a review. *Science of the total environment*, 338(1), 3-14.

Kanao, T., Kamimura, K., & Sugio, T. (2007). Identification of a gene encoding a tetrathionate hydrolase in< i> Acidithiobacillus ferrooxidans</i>. *Journal of biotechnology*, 132(1), 16-22.

Kappler, U., & Dahl, C. (2001). Enzymology and molecular biology of prokaryotic sulfite oxidation1. *FEMS microbiology letters*, 203(1), 1-9.

Karavaiko, G. I., Dubinina, G. A., & Kondrat'eva, T. F. (2006). Lithotrophic microorganisms of the oxidative cycles of sulfur and iron. *Microbiology*, 75(5), 512-545.

Kelly, D. P., & A. P. Wood. (2000) .Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *International journal of systematic and evolutionary microbiology*, 50, 511–516.

Keller, J., Owens, C. T., Lai, J. C., & Devaud, L. L. (2005). The effects of 17 beta-estradiol and ethanol on zinc- or manganese-induced toxicity in SK–N–SH cells. *Neurochemistry International* 46, 293–303.

Khan, S., Ahmad, I., Shah, M. T., Rehman, S., & Khaliq, A. (2009). Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management*, 90(11), 3451-3457.

Kishimoto, N., Kosako, Y., & Tano, T. (1991). Acidobacterium capsulatum gen. nov., sp. nov.: an acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. *Current microbiology*, 22(1), 1-7.

Klatt, C. G., Liu, Z., Ludwig, M., Kühl, M., Jensen, S. I., Bryant, D. A., & Ward, D. M. (2013). Temporal metatranscriptomic patterning in phototrophic Chloroflexi inhabiting a microbial mat in a geothermal spring. *The ISME journal*, 7(9), 1775-1789.

Kolmert, A., & Johnson, D.B. (2007). Remediation of acidic waste waters using immobilised, acidophilic sulfate-reducing bacteria. *Journal of Chemical Technology and Biotechnology*, 76(8), 836-843.

Kuenen, J.G., & Beudeker, R. F. (1982). Microbiology of Thiobacilli and other sulphur oxidising autotrophs mixotrophs and heterotrophs. In *Sulphur bacteria* (pp. 473-497). University Press, Cambridge.

Küsel, K., Dorsch, T., Acker, G., & Stackebrandt, E. (1999). Microbial reduction of Fe (III) in acidic sediments: isolation of Acidiphilium cryptum JF-5 capable of coupling the reduction of Fe (III) to the oxidation of glucose. *Applied and environmental microbiology*, 65(8), 3633-3640.

Lee, K. Y., Kim, K. W., & Kim, S. O. (2010). Geochemical and microbial effects on the mobilization of arsenic in mine tailing soils. *Environmental Geochemistry and Health* 32, 31–44.

Lee, D. J., Lee, C. Y., & Chang, J. S. (2012). Treatment and electricity harvesting from sulfate/sulfide-containing wastewaters using microbial fuel cell with enriched sulfate-reducing mixed culture. *Journal of hazardous materials*, 243, 67-72.

LeGall, J., & Fauque, G. (1988). Dissimilatory reduction of sulphur compounds. In *Biology of Anaerobic Microorganisms* ed. Zehnder, A. J. B. (pp. 587–639). Wiley, New York.

Lens, P. N., & Pol, L. H. (Eds.). (2000). Environmental technologies to treat sulfur pollution: principles and engineering. IWA Publishing.

Liu, Y., Beer, L. L., & Whitman, W. B. (2012). Sulfur metabolism in archaea reveals novel processes. *Environmental microbiology*, 14(10), 2632-2644.

Löffler, F. E., Yan, J., Ritalahti, K. M., Adrian, L., Edwards, E. A., Konstantinidis, K. T., Spormann, A. M., et al: (2013). Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi. *International journal of systematic and evolutionary microbiology*, 63(Pt 2), 625-635.

Loy, A., Duller, S., Baranyi, C., Mussmann, M., Ott, J., Sharon, I., Béjà, O., Le Paslier, D., Dahl, C., & Wagner, M. (2009). Reverse dissimilatory sulfite reductase as phylogenetic marker for a subgroup of sulphur-oxidizing prokaryotes. *Environmental Microbiology*, 11(2), 289-299.

McInerney, M. J., & Bryant, M. P. (1981). Anaerobic degradation of lactate by syntrophic associations of Methanosarcina barkeri and Desulfovibrio species and effect of H2 on acetate degradation. *Applied and environmental microbiology*, 41(2), 346-354.

Madigan, M. T. (2003). Anoxygenic phototrophic bacteria from extreme environments. *Photosynthesis research*, 76(1-3), 157-171.

Madigan, M.T, Martinko, J.M., & Brock, T.D. (2006). *Brock Biology of Microorganisms 11/e 2006 Pearson Prentice Hall, Inc*, 11/e Upper Saddle River, NJ : Pearson Prentice Hall.

Malik, S., Beer, M., Megharaj, M., & Naidu, R. (2008). The use of molecular techniques to characterize the microbial communities in contaminated soil and water. *Environment International*, 34(2), 265-276.

Manz, W., Eisenbrecher, M., Neu, T. R., & Szewzyk, U. (1998). Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated in situ probing with specific 16 S rRNA targeted oligonucleotides. *FEMS Microbiology Ecology*, 25(1), 43-61.

Mara, D. (2008). Waste stabilization ponds: a highly appropriate wastewater treatment technology for Mediterranean countries. In *Efficient Management of Wastewater* (pp. 113-123). Springer Berlin Heidelberg.

Martins, N., Bollinger, C., Harper, R. M., & Ribeiro, R. (2009). Effects of acid mine drainage on the genetic diversity and structure of a natural population of *Daphnia longispina*. *Aquatic toxicology*, 92(2), 104-112.

Masau, R. J., Oh, J. K., & Suzuki, I. (2001). Mechanism of oxidation of inorganic sulphur compounds by thiosulphate-grown *Thiobacillus thiooxidans*. *Canadian journal of microbiology*, 47(4), 348-358.

McCarthy, T. S. (2011). The impact of acid mine drainage in South Africa.*South African Journal of Science*, 107(5-6), 01-07.

Men, E., Wilson, P., Siemering, K., & Forrest, S. (2008). *Sanger DNA Sequencing 1/e 2008* (pp 3-11). Wiley-VCH.

Mine Environment Neutral Drainage Program. (1990). Assessment of Existing Natural Wetlands affected by low pH, Metal Contaminated Seepages (Acid Mine Drainage), MEND Report No. 3.12.1a, Natural Resources Canada, Ottawa.

Mine Environment Neutral Drainage Program. (1993). Treatment of Acidic Seepages using Wetland Ecology and Microbiology: Overall Program Assessment, MEND Report No. 3.11.1, Natural Resources Canada, Ottawa.

Moura, A., Tacao, M., Henriques, I., Dias, J., Ferreira, P., & Correia, A. (2009). Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR–DGGE analysis. *Microbiological Research*, 164(5), 560-569.

Mthembu, M. S., Odinga, C. A., Swalaha, F. M., & Bux, F. (2013). Review Constructed wetlands: A future alternative wastewater treatment technology. *African Journal of Biotechnology*, 12(29), 4542-4553.

Müller, F. H., Bandeiras, T. M., Urich, T., Teixeira, M., Gomes, C. M., & Kletzin, A. (2004). Coupling of the pathway of sulphur oxidation to dioxygen reduction: characterization of a novel membrane-bound thiosulphate quinine oxidoreductase. *Molecular microbiology*, 53(4), 1147-1160.

Muyzer, G., & Stams, A. J. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nature Reviews Microbiology*, 6(6), 441-454.

Nagpal, S., Chuichulcherm, S., Livingston, A., & Peeva, L. (2000). Ethanol utilization by sulphate-reducing bacteria: an experimental and modeling study. *Biotechnology and Bioenginerring*, 16(5), 533–43.

Nairn, R. W., Hedin, R. S., & Watzlaf, G.R. (1992). *Generation of alkalinity in an anoxic limestone drain*. Proc. of the 9th Annual National Meeting of the American Society for Surface Mining and Reclamation, Duluth, Minnesota.

Netto, E., Madeira, R. A., Silveira, F. Z., Fiori, M. A., Angioleto, E., Pich, C. T., & Geremias, R. (2013). Evaluation of the toxic and genotoxic potential of acid mine drainage using physicochemical parameters and bioassays. *Environmental toxicology and pharmacology*, 35(3), 511-516.

Nordstrom, D. K., & Alpers, C. N. (1999a). Negative pH, efflorescent mineralogy, and consequences for environmental restoration at the Iron Mountain Superfund site, California. *Proceedings of the National Academy of Sciences*, 96(7), 3455-3462.

Nordstrom, D. K., & Alpers, C. N. (1999b). Geochemistry of acid mine waters. *The Environmental geochemistry of mineral deposits. part a: processes, techniques, and health issues*, 6, 133-160.

Offre, P., Spang, A., & Schleper, C. (2013). Archaea in Biogeochemical Cycles. *Annual Review of Microbiology*, 67, 437-457.

Olem, H., & Unz, R. F. (1977). Acid mine drainage treatment with rotating biological contactors. *Biotechnology and bioengineering*, 19(10), 1475-1491.

Oliveira, T. F., Vonrhein, C., Matias, P. M., Venceslau, S. S., Pereira, I. A., & Archer, M. (2008). The crystal structure of Desulfovibrio vulgaris dissimilatory sulfite reductase bound to DsrC provides novel insights into the mechanism of sulfate respiration. *Journal of Biological Chemistry*, 283(49), 34141-34149.

Office of national assessment (ONAS). (2009). Annual report of the National Office of Sanitation. National Office of Sanitation, Tunisia.

Overmann, J., & Tuschak, C. (1997). Phylogeny and molecular fingerprinting of green sulfur bacteria. *Archives of microbiology*, 167(5), 302-309.

Pattaragulwanit, K., Brune, D. C., Trüper, H. G., & Dahl, C. (1998). Molecular genetic evidence for extracytoplasmic localization of sulfur globules in Chromatium vinosum. *Archives of microbiology*, 169(5), 434-444.

Peplow, D., & Edmonds, R. (2005). The effects of mine waste contamination at multiple levels of biological organization. *Ecological Engineering*, 24, 101–119.

Pfennig, N. (1989). Ecology of phototrophic purple and green sulfur bacteria. *Autotrophic Bacteria*, 97-116.

Pfennig, N., & Trüper, H. G. (1992). The family Chromatiaceae. In *The Prokaryotes*, 2nd edn. (pp. 3584-3592). New York: Springer.

Pott, A.S., & Dahl, C. (1998). Sirohaem sulfite reductase and other proteins encoded by genes at the dsr locus of Chromatium vinosum are involved in the oxidation of intracellular sulphur. *Microbiology*, 144(7), 1881-1894**.**

Prange, A., Chauvistré, R., Modrow, H., Hormes, J., Trüper, H. G., & Dahl, C. (2002). Quantitative speciation of sulfur in bacterial sulfur globules: X-ray absorption spectroscopy reveals at least three different species of sulfur.*Microbiology*, 148(1), 267-276.

Pruden, A., Pereya, L.P., Hijbel, S.R., Inman, L.Y., Kashani, N., Reardon, K., & Reisman, D. (2006). Microbiology of sulfate-reducing passive treatment systems, 7th ICARD. St. Louis, MO. Published by ASMR.

Pulles, W., Rose, P., Coetser, L., & Heath, R. (2003). Development of Integrated Passive Water Treatment Systems for the Treatment of Mine Waters, Proceedings of ICARD Conference, 12-18 July, Cairns, Australia.

Pulles, W., & Heath, R. (2009). The evolution of passive mine water treatment technology for sulphate removal, Golder Associates Africa (Pty) Ltd, PO Box 6001, Halfway House, 1685, South Africa. International Mine Water Conference.

Rabus, R., Hansen, T. A., & Widdel, F. (2006). Dissimilatory sulfate-and sulfur-reducing prokaryotes. In *The prokaryotes* (pp. 659-768). Springer New York.

Rohwerder, T., & Sand, W. (2003a). The sulfane sulphur of persulfides is the actual substrate of the sulphur-oxidizing enzymes from *Acidithiobacillus* and *Acidiphilium* spp. *Microbiology*, 149, 1699–1709.

Rohwerder, T., Gehrke, T., Kinzler, K., & Sand, W. (2003b). Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied Microbiology and Biotechnology*, 63(3), 239-248.

Rohwerder, T., & Sand, W. (2007). Oxidation of inorganic sulfur compounds in acidophilic prokaryotes. *Engineering in Life Sciences*, 7(4), 301-309.

Sahm, K., Knoblauch, C., & Amann, R. (1999). Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine arctic sediments. *Applied and environmental microbiology*, 65(9), 3976-3981.

Sánchez-Andrea, I., Triana, D., & Sanz, J. L. (2012). Bioremediation of acid mine drainage coupled with domestic wastewater treatment. *Water Science & Technology*, 66(11), 2425-2431.

Sand, W., Gerke, T., Hallmann, R., & Schippers, A. (1995). Sulfur chemistry, biofilm, and the (in) direct attack mechanism—a critical evaluation of bacterial leaching. *Applied Microbiology and Biotechnology*, 43(6), 961-966.

Sand, W., Gehrke, T., Jozsa, P. G., & Schippers, A. (2001). (Bio) chemistry of bacterial leaching—direct vs. indirect bioleaching. *Hydrometallurgy*, 59(2), 159-175.

Sander, J., Engels-Schwarzlose, S., & Dahl, C. (2006). Importance of the DsrMKJOP complex for sulfur oxidation in Allochromatium vinosum and phylogenetic analysis of related complexes in other prokaryotes. *Archives of microbiology*, 186(5), 357-366.

Sasikala, C., & Ramana, C. V. (1997). Biodegradation and metabolism of unusual carbon compounds by anoxygenic phototrophic bacteria. *Advances in microbial physiology*, 39, 339-377.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74(12), 5463-5467.

Schippers, A., Jozsa, P., & Sand, W. (1996). Sulfur chemistry in bacterial leaching of pyrite. *Applied and Environmental Microbiology*, 62(9), 3424-3431.

Schippers, A., & Sand, W. (1999). Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. *Applied and Environmental Microbiology*, 65(1), 319-321.

Schippers, A. (2007). Microorganisms involved in bioleaching and nucleic acid-based molecular methods for their identification and quantification. In *Microbial processing of metal sulfides* (pp. 3-33). Springer Netherlands.

Segerer, A., Stetter, K. O., & Klink, F. (1985). Two contrary modes of chemolithotrophy in the same archaebacterium.

Senko, J. M., Wanjugi, P., Lucas, M., Bruns, M. A., & Burgos, W. D. (2008). Characterization of Fe (II) oxidizing bacterial activities and communities at two acidic Appalachian coalmine drainage-impacted sites. *The ISME journal*, *2*(11), 1134-1145.

Seviour, R. J., & Blackall, L. L. (1999). *The Microbiology of Activated Sludge*. London: Kluwer.

Skousen, J., Lilly, R. and Hilton, T. (1991). Special chemicals for treating acid mine drainage. *Green Lands,* 34-41.

Skousen, J. (1997). Overview of passive systems for treating acid mine drainage. *Green Lands*, 27(4), 34-43.

Sorokin, D. Y., Lysenko, A. M , Mityushina, L. L., Tourova, T. P., Jones, B. E , Rainey, F. A., L., Robertson, A., & Kuenen, J.G. (2001). *Thioalkalimicrobium aerophilum* gen. nov., sp. nov., and *Thioalkalimicrobium sibericum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., *Thioalkalivibrio nitratis* sp. nov., and *Thioalkalivibrio denitrificans* sp. nov., novel

obligately alkaliphilic and obligately chemolithoautotrophic sulphur-oxidizing bacteria from soda lakes. *International Journal of Systemic and Evolutionary Microbiology*, 51, 565–580.

Stams, A. J. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek*, 66(1-3), 271-294.

Steed, V.S., Suidan, M.T., Gupta, M., Miyarahara, T., Acheson, C.M., & Sayles, G.D. (2000). Development of a sulphate-reducing biological process to remove heavy metals from acid mine drainage. *Water Environmental Resources*, 72(5), 530–535.

Steinberg, L. M., & Regan, J.M. (2008). Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. *Applied and environmental microbiology*, 74(21), 6663-6671.

Stetter, K. O., Fiala, G., Huber, G., Huber, H., & Segerer, A. (1990). Hyperthermophilic micro-organisms, *FEMS Microbiology Rev*iew, 75, 117–124.

Steudel, R., Holdt, G., Göbel, T. & Hazeu, W., 1987, Chromatographic separation of higher polythionates SnO62- (*n* = 3…22) and their detection in cultures of *Thiobacillus ferrooxidans*: molecular composition of bacterial sulphur secretion, *Angewandte Chemie International Edition in English*, 26(2), 151-153.

Stoltz, E. (2003). *Phytostabilisation: use of wetland plants to treat mine tailings*. Doctoral thesis. Stockholm University, Stockholm, 1–45.

Stottmeister, U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A., & Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances*, 22(1), 93-117.

Strohl, W. R. (1989). Genus I. Beggiatoa, p. 2091–2097. Int J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins, Baltimore, Md.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011a). Novel passive co-treatment of acid mine drainage and municipal wastewater. *Journal of environmental quality*, 40(1), 206-213.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011b). Alkalinity generation in a novel multi-stage high-strength acid mine drainage and municipal wastewater passive co-treatment system. *Mine Water and the Environment*, 30(1), 47-53.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011c). Biochemical oxygen demand and nutrient processing in a novel multi-stage raw municipal wastewater and acid mine drainage passive co-treatment system. *Water Research*, 45(3), 1079-1086.

Strosnider, W.H.I., Winfrey, B.K., Peera, R.A.M. and Nairn, R.W. (2013). Passive co-treatment of acid mine drainage and sewage: Anaerobic incubation reveals a regeneration technique and further treatment possibilities. *Ecological Engineering*, 61, 268– 273.

Stottmeister, U., Wiebner, A., Kuschk, P. and Kappelmeyer, U. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances* 22, 93–117.

Tabita, F. R. (1988). Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiological reviews*, 52(2), 155.

Tabita, F.R. (1999). Microbial ribulose 1,5-carboxylase/oxygenase: a different perspective. *Photosynthesis Research.* 60, 1–28.

Takacs, C.D., Reysenbach, A.L., & Boone, D.R. (2001). Archaeal Ecology,. *Encyclopedia of Life Sciences*.

Tamás, L., Huttova, J., Mistrik, I., Simonovicova, M., & Siroka, B. (2006). Aluminum-induced drought and oxidative stress in barley roots, *Journal of Plant Physiology*, *163*, 781–784.

Tang, K.H., Barry, K., Chertkov, O., Dalin, E., Han, C.S., Hauser, L.J., Honchak, B.M., Karbach, L.E., Land, M.L., Lapidus, A., Larimer, F.W., Mikhailova, N., Pitluck, S., Pierson, B.K., & Blankenship, R.E. (2011). Complete genome sequence of the filamentous anoxygenic phototrophic bacterium Chloroflexus aurantiacus, *BMC Genomics*, 12, 334.

Thauer, R.K. (2007). A fifth pathway of carbon fixation. *Science*, 318, 1732–1733.

Van der Merwe, W., & Lea, I. (2003). *Towards sustainable mine water treatment at Grootvlei Mine.* Proceedings of the 8th International Congress on Mine Water and the Environment; 2003 Oct 19–22; Johannesburg, South Africa. Armstrong D, de Viviers AB, Klieinmann RLP, McCarthy TS, Norton, PJ, editors. International Mine Water Association, pp. 25–36.

Van Gemerden, H., & Mas, J. (1995). Ecology of phototrophic sulfur bacteria. In *Anoxygenic photosynthetic bacteria* (pp. 49-85). Springer Netherlands.

Wakai, S., Kikumoto, M., Kanao, T., & Kamimura, K. (2004). Involvement of sulfide: quinone oxidoreductase in sulfur oxidation of an acidophilic iron-oxidizing bacterium, Acidithiobacillus ferrooxidans NASF-1. *Bioscience, biotechnology, and biochemistry*, 68(12), 2519-2528.

Ward, D.M., & Winfrey, M.R. (1985). Interactions between methanogenic and sulfate-reducing bacteria in sediments. *Advances in aquatic microbiology*, 3, 141-179.

White, D., Drummond, J. T., & Fuqua, C. (1995). *The physiology and biochemistry of prokaryotes* (pp. 12-21). New York: Oxford University Press.

White, C., & Gadd, G. M. (1996). A comparison of carbon/energy and complex nitrogen sources for bacterial sulphate-reduction: potential applications to bioprecipitation of toxic metals as sulphides. *Journal of industrial microbiology*, 17(2), 116-123.

Widdel, F. (1988). Microbiology and ecology of sulfate-and sulfur-reducing bacteria. *Biology of anaerobic microorganisms*, 469-585.

Widdel, F., & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes* (pp. 3352-3378). Springer New York.

Wieder, R. K. (1989). A survey of constructed wetlands for acid coal mine drainage treatment in the eastern United States. *Wetlands*, 9(2), 299-315.

Wilderer, P. A., Arnz, P., & Arnold, E. (2000). Application of biofilms and biofilm support materials as a temporary sink and source. *Water, air, and soil pollution*, 123(1-4), 147-158.

Winfrey, M. R., & Zeikus, J. G. (1977). Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. *Applied and Environmental Microbiology*, 33(2), 275-281.

Xie, J., He, Z., Liu, Xinxing, Liu, Xueduan, Van Nostrand, J. D., Deng, Y., Wu, L., Zhou, J., & Qiu, G. (2011). GeoChip-based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage. *Applied and environmental microbiology*, 77(3), 991-999.

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H., & Kamagata, Y. (2006). Anaerolinea thermolimosa sp. nov., Levilinea saccharolytica gen. nov., sp. nov. and Leptolinea tardivitalis gen. nov., sp. nov., novel filamentous anaerobes, and description of the

new classes Anaerolineae classis nov. and Caldilineae classis nov. *International Journal of Systematic and Evolutionary Microbiology*, 56, 1331–1340.

Yoda, M., Kitagawa, M., & Miyaji, Y. (1987). Long Term Competition Between Sulfate-reducing and Methane-producing Bacteria for Acetate in Anaerobic Biofilm, *Water Resources (G.B.)*, 21, 1547.

Zhang, J., Zhang, Y., Chang, J., Quan, X., & Li, Q. (2013). Biological sulfate reduction in the acidogenic phase of anaerobic digestion under dissimilatory Fe (III) - reducing conditions, *Water Research*, 47, 2033-2040.

Zarzycki, J., Brecht, V., Müller, M., & Fuchsa, G. (2009). Identifying the missing steps of the autotrophic 3-hydroxypropionate CO2 fixation cycle in Chloroflexus aurantiacus. *Proceedings of the National Academy of Sciences USA*, 21317–21322.

Zdyb, L. (1999). Microbial sulfate reduction as a method of passive treatment of acid mine drainage using undefined carbon sources. University of Pretoria. South Africa, pp. 67.

Zillig, W., Yeats, S., Holz, I., Böck, A., Gropp, F., Rettenberger, M., & Lutz, S. (1985). Plasmid-related anaerobic autotrophy of the novel archaebacterium Sulfolobus ambivalens.

Zhou, Q., Chen, Y., Yang, M., Li, W., & Deng, L. (2013). Enhanced bioremediation of heavy metal from effluent by sulfate-reducing bacteria with copper–iron bimetallic particles support. *Bioresource technology*, 136, 413-417.

Chapter 3

The design and use of synthetic domestic waste water sludge medium to determine the optimum ratio of acid mine drainage to sludge for maximum sulphate reduction.

1. Introduction

Industrial and mine wastewater is generally referred to as acid mine drainage (AMD) and is characteristically acidic of nature containing sulphur, pyrite and other heavy metals (Geremias *et al.*, 2003). When untreated, AMD undergoes biological and chemical changes when exposed to dissolved oxygen, water and micro-organisms (Nordstrom & Alpers, 1999; Benner *et al.*, 2000; Baker & Banfield, 2003; Johnson & Hallberg, 2003) and is consequently converted into an environmental pollutant posing a threat to the environment (Peplow & Edmonds, 2005; Lee *et al.*, 2010) and human health (Keller *et al.*, 2005).

As specified in the South African water quality guidelines (DWAF, 1996) a sulphate level exceeding 200 mg/l is unsuitable for use for domestic purposes. However, AMD may contain sulphate concentrations as high as 1500 mg/L (Van der Merwe & Lea, 2003). One of the treatment methods for the reduction of sulphate in AMD relies on sulphate reducing bacteria (SRB) in bioreactors (Garcia *et al.*, 2001) and phytoremediation through constructed wetlands (Stottmeister *et al.*, 2003; Collins *et al.*, 2005; Khan *et al.*, 2009). SRB uses inorganic sulphate as a terminal electron acceptor obtained by oxidation of carbon sources and the reduction of sulphate or molecular hydrogen to hydrogen sulfide (LeGall & Fauque, 1988).

The use of sulphate reducing bacteria for the reduction of sulphates present in AMD relies on the provision of a suitable environment (Garcia *et al.*, 2001; Kappler & Dahl, 2001; Burns *et al.*, 2012; Sánchez-Andrea *et al.*, 2012). An example of such an environment is a typical passive sulphate reducing bioreactor that is comprised of a basin-like structure which contains a suitable organic substrate and a sulphate reducing bacterial consortium. The AMD to be treated enters through an inflow after seeping through the organic matter/ microbial inoculum to reduce the sulphate in the system and increase the alkalinity to neutralise the AMD. The treated water may in some cases then be released into a wetland for further treatment (Gusek, 2002).

Phytoremediation is another option for treating AMD and relies on hydrophilic plants and the associated micro-organisms for the removal of AMD contaminants. Constructed wetlands is an example of such a phytoremediation option (Stottmeister *et al.*, 2003; Collins *et al.*, 2005; Khan *et al.*, 2009; Allende *et al.*, 2011) for the passive, non-chemical treatment of AMD.

The treatment of municipal domestic wastewater, although a necessity, is a task many developing countries fail to do due to cost and energy resources required for the treatment of municipal wastewater (Muga & Mihelcic, 2008; Strosnider *et al.*, 2011a). As a result, untreated municipal wastewater is often released into natural water resources, reducing the quality of available water for domestic use (Gadgil, 1998; Kivaisi, 2001; (Strosnider *et al.*, 2011b). The co-treatment of AMD and municipal wastewater has become a treatment option of interest as the simultaneous treatment of municipal wastewater and AMD allows a reduction in treatment costs (Strosnider *et al.*, 2011b; Strosnider *et al.*, 2011c; Strosnider *et al.*, 2013).

The chemical composition of domestic waste varies (Al-Salem, 1987; Mohammed *et al.*, 2012) and therefore synthetic media simulating domestic waste have been formulated for research purposes (Hiraishi *et al*. 1998; Mazumder, 2010). However these formulae vary in composition due to different applications in the research studies. Therefore the aim of this study was to formulate new synthetic domestic waste water sludge to determine the optimum ratio of sludge to AMD for maximum sulphate reduction.

2. Materials and methods

2.1 Formulation of synthetic domestic wastewater sludge media

For the formulation of the synthetic anaerobic domestic wastewater sludge (SDWWS), only the nutritional value of the anaerobic domestic wastewater sludge was of interest and not the specific chemical composition itself, hence the exclusion of most trace metals (Stover *et al.*, 1976; Alloway & Jackson, 1991). The chemical oxygen demand (COD), biological oxygen demand (BOD), sulphate concentration and pH determined for anaerobic domestic wastewater sludge were used as the nutrient parameters as described below.

2.1.1 Chemical analysis of anaerobic domestic wastewater sludge

Anaerobic domestic wastewater sludge was obtained from the anaerobic digester tank at the Pniel wastewater treatment plant situated on the outer skirt of Stellenbosch by collecting samples in 5 L plastic containers. These containers were kept at room temperature (22°C) until chemical analyses were conducted within 24 h. The COD and sulphate concentrations were determined by using the Merck Spectroquant Pharo 300 and cell test kits according to the recommended protocol. A BOD 16S kit from Oxitop was used to determine the BOD and pH was determined by using a digital pH meter (PCTestr 35 Multi-Parameter).

2.1.2 Composition of the synthetic domestic wastewater sludge media

Vegetable extract (Sigma-Aldrich (Pty) Ltd., Aston Manor, South Africa) and meat extract (Sigma-Aldrich) served as the basis of the synthetic media as it incorporates the protein, carbohydrate and fat content. The rest of the components included sodium chloride (Sigma-Aldrich), potassium phosphate (Sigma-Aldrich), urea (Sigma-Aldrich), ammonium chloride (Sigma-Aldrich), iron sulphate (Sigma-Aldrich), magnesium sulphate (Sigma-Aldrich) and glucose (Sigma-Aldrich) (Table 1). Four different ratios of the mentioned components were prepared and chemical analysis including COD, BOD, sulphate concentrations and pH was performed as described before. The medium that compared best to the chemical analysis of SDWWS was selected for further optimization. The optimized SDWWS media was then used for further studies.

Table 1. Composition of the four SDWWS media

Component	**Medium 1**	**Medium 2**	**Medium 3**	**Medium 4**
Meat extract (g/L)	20	0.6	10	40
Vegetable extract (g/L)	35	0.06	10	20
Sodium chloride (g/L)	1	0.2	0.2	1.3
Potassium phosphate (g/L)	1	0.1	1	1
Urea (g/L)	8	0.1	10	10
Ammonium chloride (g/L)	1	0.5	0	0
Iron sulphate (g/L)	1	0.1	1	1
Magnesium sulphate (g/L)	1	0.1	1	1
Glucose (g/L)	0	0.5	5	3

2.2 Determining the optimal AMD to SDWWS ratio

2.2.1 Experimental design for the anaerobic treatment of AMD

Medical drip bags (1 L) (Stelmed, Stellenbosch, South Africa) served as small anaerobic bioreactors. Acid mine drainage sampled from an Exxaro Coal mine was couriered overnight in 5 L plastic containers and stored at room temperature (20-21°C) until use. Three ratios of AMD and the selected SDWWS (as described in section 2.1.2) were prepared to a final volume of 900 mL in the bioreactors and the pH adjusted to 7.5 with 5 mM NaOH solution

where needed (Table 2). The bioreactors were then incubated upright in a dimly lit enclosed environment at room temperature (20-21°C) for 90 d (Figure 1). Mixtures of AMD and sterile distilled water (dH_2O) in the ratios of 1:1, 1:2 and 2:1 served as experimental controls. The trial was run in triplicate and repeated.

Table 2. Ratios of the controls and synthetic domestic waste water sludge (SDWWS) to AMD

Ratio	**Composition**		
	dH_20 (mL)	**AMD (mL)**	**SDWWS (mL)**
AMD control 1:2	300	600	0
AMD control 1:1	450	450	0
AMD control 2:1	600	300	0
Ratio 1 1:2	0	600	300
Ratio 2 1:1	0	450	450
Ratio 3 2:1	0	300	600
Medium control 1:2	300	0	600
Medium control 1:1	450	0	450
Medium control 2:1	600	0	300

From here on the 1:2 ratio will be referred to as Ratio 1, the 1:1 ratio referred to as Ratio 2 and the 2:1 as Ratio 3.

Figure 1. The bioreactors containing different ratios of AMD and SDWWS on Day 0 of incubation.

2.2.2 Microbial inoculum used in the bioreactors

Anaerobic domestic wastewater sludge obtained from the anaerobic digester tank at the Pniel wastewater treatment plant was used as microbial inoculum. Samples were collected in 5 L containers and left overnight at 21°C. Thereafter the bioreactors containing the SDWWS:AMD ratios (Table 2) were inoculated with 10 mL domestic wastewater sludge.

2.2.3 Chemical analyses of the different ratios SDWWS to AMD

The COD and sulphate concentrations of the different ratios of SDWWS to AMD were determined on days 1 and 90 of the trails as previously described.

3. Results and Discussion

3.1 Formulation of synthetic anaerobic domestic wastewater sludge

The chemical analyses of the four SDWWS media are indicated in Table 3. The COD and BOD of Medium 3 were 2600 mg/L and 330, respectively, and compared best to the COD (3650 mg/L) and BOD (320) of anaerobic domestic wastewater. The concentrations of components in Medium 3 was further optimised by increasing the concentration of meat extract and decreasing the concentrations of vegetable extract, sodium chloride, magnesium sulphate, potassium phosphate, iron sulphate, urea and glucose (Table 4). The COD of the optimised synthetic DWWS medium was 3646 mg/L, the BOD was 317 and the pH 6.9.

Table 3. The chemical analyses conducted on the anaerobic domestic wastewater sludge.

Sample	COD (mg/L)	Std Dev	BOD	pH
Anaerobic domestic wastewater sludge sample	3650	5.2	320	6.9
Medium 1	15 500	6.4	3500	7.0
Medium 2	1900	2.4	200	7.1
Medium 3	2600	4.3	330	6.8
Medium 4	17 000	6.1	3600	6.5

Table 4. Composition of optimised synthetic domestic wastewater sludge

Component	Mass mg/L
Meat extract	2182
Vegetable extract	218
NaCl	72.7
$MgSO_4$	182
KH_2PO_4	145
$FeSO_4$	36
Glucose	182

3.2 Chemical analyses of the different ratios of SDWWS to AMD

The COD and sulphate concentrations of all the controls decreased between 0.83 % and 6.54 %. The media control values are not indicated in the graphs (Figures 2 & 3). A decrease of between 1.67 % and 6.25 % in sulphate content in the controls can possibly be contributed to the precipitation of metal sulphates, including iron sulfide. The reduction in both COD and sulphate levels in Ratio 1 and Ratio 3 were similar. The highest reduction of 86.76 % in COD and 99.22 % in sulphate content were obtained in Ratio 2 (Figures 2 & 3). These results are in contradiction with the study conducted by Strosnider *et al.*, (2011a) where optimum results were achieved by an AMD: sewage ratio of 1:2. However, Strosnider *et al.*, (2011a) focussed on the ratio of AMD to sewage that resulted in the highest reduction of metal concentrations during treatment of AMD and sewage.

The COD and sulphate content showed a decline after 14 d and reached a plateau after 90 d, 61 days less than a study conducted by Strosnider *et al.* (2013). This could be attributed to the smaller volumes of AMD treated in this study. This study will need to be conducted on a larger scale to determine the impact of larger bioreactors on the treatment results. Poinapen and co-workers (2009) investigated the use of a semi-flow through anaerobic sludge bed reactors with sewage as carbon source. The trial was conducted at 35°C and delivered a sulphate reduction of 90 % with a 14 h retention time, compared to the ≈99 % in this study (Figure 3).

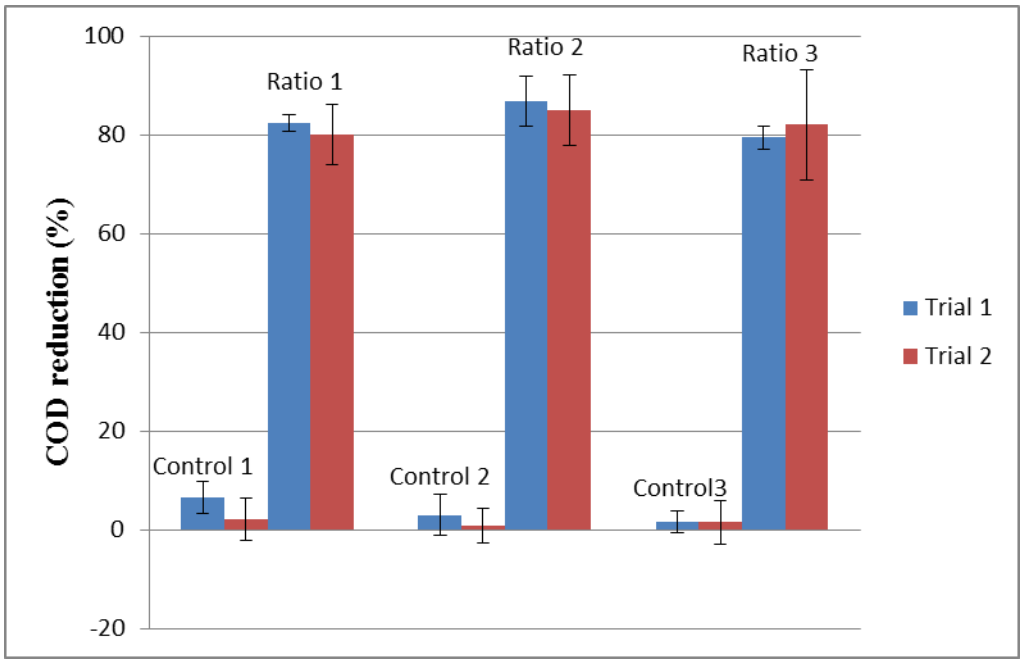

Figure 2: The average percentage reduction in COD after the 90 d incubation period for Trials 1 and 2.

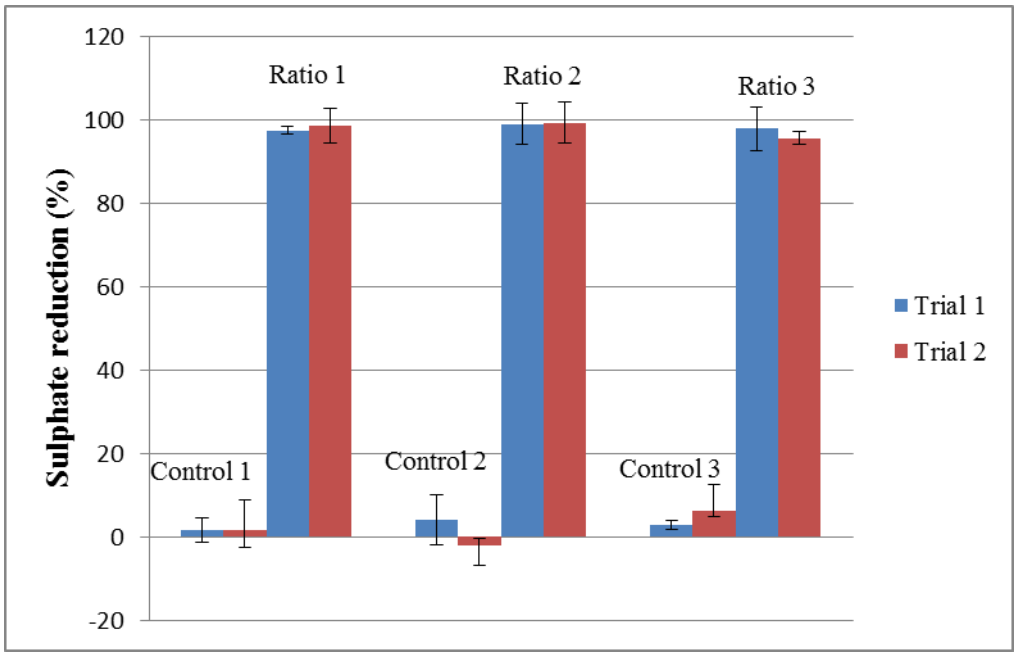

Figure 3. The average percentage reduction in sulphates after the 90 d incubation period for Trials 1 and 2.

4. Conclusion

Synthetic media simulating domestic wastewater sludge was suitable as an alternative carbon source to study the use of domestic wastewater sludge for the anaerobic treatment of AMD in batch reactors. The COD and sulphate content of the AMD were reduced by >85% and >98% by bioreactors containing a 1:1 AMD:SDWWS ratio. The design of synthetic anaerobic domestic wastewater sludge also excluded the variables found in anaerobic domestic wastewater sludge collected at wastewater plants (Henze, 2008; Abbas *et al.*, 2011; Snaidr *et al.*, 1997; Boon *et al.*, 2002; Juretschko *et al.*, 2002). However, as only small volumes of AMD and domestic wastewater sludge were treated per bioreactor, the results obtained during this study may differ in the treatment of larger volumes of wastewater. In future studies the volume of AMD to be treated should be up-scaled to determine the efficiency of a 1:1 ratio of SDWWS:AMD in a bioreactor for COD and sulphate content reduction.

5. References

Abbas, A. H., Ibrahim, A. B. A., Nor, M. F. M., & Aris, M. S. (2011). Characterization of Malaysian domestic sewage sludge for conversion into fuels for energy recovery plants. In *National Postgraduate Conference (NPC)*, pp. 1-4.

Allende, K. L., Fletcher, T. D., & Sun, G. (2011). Enhancing the removal of arsenic, boron and heavy metals in subsurface flow constructed wetlands using different supporting media. *Water Science and Technology*, 63(11), 2612-2618.

Alloway, B.J. & Jackson, A.P. (1991). The behaviour of heavy metals in sewage sludge-amended soils. *The Science of the Total Environment*, 100, 151-176.

Al-Salem, S.S. (1987). Evaluation of the Al Samra waste stabilization pond system and its suitability for unrestricted irrigation. Paper prepared for the Land and Water Development Division, FAO, Rome irrigation.

Baker, B. J., & Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, 44(2), 139-152.

Benner, S.G., Gould, W.D., & Blowes, D.W. (2000). Microbial populations associated with the generation and treatment of acid mine drainage. *Chemical Geology*, 169, 435-448.

Boon, N., De Windt, W., Verstraete, W., & Top, E.M. (2002). Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiology Ecology*, 39, 101-112.

Burns, A. S., Pugh, C. W., Segid, Y. T., Behum, P. T., Lefticariu, L., & Bender, K. S. (2012). Performance and microbial community dynamics of a sulfate-reducing bioreactor treating coal generated acid mine drainage. *Biodegradation*, 2(3), 415-429.

Collins, B. S., Sharitz, R. R., & Coughlin, D. P. (2005). Elemental composition of native wetland plants in constructed mesocosm treatment wetlands. *Bio-resource Technology*, 96(8), 937–948.

DWAF, Water quality guidelines for South Africa: First Edition 1996.

Gadgil, A. (1998). Drinking water in developing countries. *Annual Review of Energy and the Environment*, 23(1), 253-286.

Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L., & Gonzalez, F. (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering*, 14(9), 997-1008.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Favere, V. T., Stolberg, J., Menezes, C. T. B., & Laranjeira, M. C. M. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental technology*, 24(12), 1509-1515.

Gusek, J. J. (2002). Sulfate-reducing bioreactor design and operating issues: Is this the passive treatment technology for your mine drainage. *National Association of Abandoned Mine Land Programs, Park City, Utah*.

Henze, M. (Ed.). (2008). *Biological wastewater treatment: principles, modelling and design*. IWA publishing.

Hiraishi, A., Ueda, Y., & Ishihara, J. (1998). Quinone Profiling of Bacterial Communities in Natural and Synthetic Sewage Activated Sludge for Enhanced Phosphate Removal. *Applied and Environmental microbiology*, 64(3), 992-998.

Johnson, D. B., & Hallberg, K. B. (2003). The microbiology of acidic mine waters. *Research in Microbiology*, 154(7), 466-473.

Juretschko, S., Loy, A., Lehner, A., & Wagner, M. (2002). The Microbial Community Composition of a Nitrifying-Denitrifying Activated Sludge from an Industrial Sewage Treatment Plant Analyzed by the Full-Cycle rRNA Approach. *Systematic and Applied Microbiology*, 25, 84–99.

Kappler, U., & Dahl, C. (2001). Enzymology and molecular biology of prokaryotic sulfite oxidation1. *FEMS microbiology letters*, 203(1), 1-9.

Keller, J., Owens, C. T., Lai, J. C., & Devaud, L. L. (2005). The effects of 17 beta-estradiol and ethanol on zinc- or manganese-induced toxicity in SK–N–SH cells. *Neurochemistry International*, 46, 293–303.

Khan, S., Ahmad, I., Shah, M. T., Rehman, S., & Khaliq, A. (2009). Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management*, 90(11), 3451-3457.

Kivaisi, A.K. (2001). The potential for constructed wetlands for wastewater treatmentand reuse in developing countries: a review. *Ecological Engineering*, 16, 545–560.

Lee, K. Y., Kim, K. W., & Kim, S. O. (2010). Geochemical and microbial effects on the mobilization of arsenic in mine tailing soils. *Environmental Geochemistry and Health*, 32, 31–44.

LeGall, J., & Fauque, G. (1988). Dissimilatory reduction of sulphur compounds. In *Biology of Anaerobic Microorganisms* ed. Zehnder, A. J. B. pp. 587–639. Wiley, New York.

Mazumder, D. (2010). Simultaneous COD and Ammonium Nitrogen Removal from a High-strength Wastewater in a Shaft-type Aerobic Hybrid Bioreactor. *International Journal of Environmental Science and Development,* 1(4), 327-332.

Mohammed, R.A., Mohammed, A.A., & Hassan, I.H. (2012). Characteristics of Raw Domestic Sewage for Basrah City. *Basrah Journal for Engineering Science*, 60-71.

Muga, H.E., & Mihelcic, J.R. (2008). Sustainability of wastewater treatment technologies, *Journal of Environmental Management,* 88, 437–447.

Nordstrom, D. K., & Alpers, C. N. (1999). Geochemistry of acid mine waters. *The Environmental geochemistry of mineral deposits. part a: processes, techniques, and health issues*, 6, 133-160.

Peplow, D., & Edmonds, R. (2005). The effects of mine waste contamination at multiple levels of biological organization. *Ecological Engineering*, 24, 101–119.

Poinapen, J., Ekama, G.A., & Wentzel, M.C. (2009). Biological sulphate reduction with primary sewage sludge in an upflow anaerobic sludge bed (UASB) reactor -Part 4: Bed settling characteristics. *Water SA* (Online) [serial on the Internet]. 2009 Oct [cited 2014 Sep 23], 35(5), 553-560.

Sánchez-Andrea, I., Triana, D., & Sanz, J. L. (2012). Bioremediation of acid mine drainage coupled with domestic wastewater treatment. *Water Science & Technology*, 66(11), 2425-2431.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., & Schleifer, K. (1997). Phylogenetic Analysis and In Situ Identification of Bacteria in Activated Sludge. *Applied and Environmental Microbiology*, 63(7), 2884-2896.

Stottmeister, U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A., & Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances*, 22(1), 93-117.

Stover, R.C., Sommers, L.E., & Silviera, D.J. (1976). Evaluation of metals in wastewater sludge. *Journal (Water Pollution Control Federation)*, 48(9), 2164- 2175.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011a). Novel passive co-treatment of acid mine drainage and municipal wastewater. *Journal of environmental quality*, 40(1), 206-213.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011b). Alkalinity generation in a novel multi-stage high-strength acid mine drainage and municipal wastewater passive co-treatment system. *Mine Water and the Environment*, 30(1), 47-53.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011c). Biochemical oxygen demand and nutrient processing in a novel multi-stage raw municipal wastewater and acid mine drainage passive co-treatment system. *Water research*, 45(3), 1079-1086.

Strosnider, W.H.I., Winfrey, B.K., Peera, R.A.M., & Nairn, R.W. (2013). Passive co-treatment of acid mine drainage and sewage: Anaerobic incubation reveals a regeneration technique and further treatment possibilities. *Ecological Engineering*, 61, 268– 273.

Van der Merwe, W., & Lea, I. (2003). *Towards sustainable mine water treatment at Grootvlei Mine.* Proceedings of the 8th International Congress on Mine Water and the Environment. Oct 19–22, Johannesburg, South Africa.

Chapter 4

The microbial ecology of acid mine drainage treatment with synthetic domestic wastewater sludge.

1. Introduction

Acid mine drainage (AMD) is a widely studied phenomenon that refers to untreated industrial wastewater (Geremias *et al.*, 2003) and may be a threat to human and environmental health (Keller *et al.*, 2005; Lee *et al.*, 2010). AMD contains high concentrations of sulphur and heavy metals (Hughes & Gray, 2013) which result in the formation of sulphuric acid (Equations 1, 2 and 3) (Costello, 2003; Druschel *et al.*, 2004). The generation of AMD consists of a two-step process. During the first phase, ferric iron and sulphuric acid are produced (Equations 1 and 2). The second phase involves the precipitation of ferric hydroxide (Equation 3). A higher acidity leads to the complete oxidation of pyrite by ferric iron (Sand *et al.*, 2000) (Equation 4).

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2+} + 2H^+ \quad (1)$$

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½H_2O \quad (2)$$

$$Fe^{2+} + ¼O + 5/2\ H_2O \rightarrow Fe\ (OH)_3 + 2H^+ \quad (3)$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2+}\ 16H^+ \quad (4)$$

Various remediation methods for AMD have been investigated since the 1900's (Barnes & Romberger, 1968; Olem & Unz, 1977). These methods involve abiotic and biotic methods divided into active and passive systems (Johnson & Hallberg, 2005). Although the use of alkaline chemicals or limestone (Nairn *et al.*, 1992) in the abiotic treatment of AMD is convenient it is not environmentally friendly. Therefore passive biotic remediation methods such as the use of wetlands (Collins *et al.*, 2005; Khan *et al.*, 2009) and sulphate reducing bacteria (Garcia *et al.*, 2001; Zhou *et al.*, 2013) are the preferred treatment methods for AMD. The aim of such passive treatment systems is to reduce sulphate and organic matter content and to neutralize the pH of the AMD. However, these systems may require long reaction time

(Pulles & Heath, 2009) which is not always feasible in the treatment of large quantities of AMD.

A passive biotic approach to AMD treatments poorly investigated is the use of biofilms in conjunction with microbial bioreactors to decrease the sulphur content of AMD. A biofilm can be defined as a complex community of micro-organisms adhering to a surface (Characklis, 1990), forming a working unit as interaction occurs on a physical and chemical level (Costerton *et al.*, 1995; James *et al.*, 1995). Biofilms have been utilized in wastewater remediation processes such as a biofilm upflow sludge blanket (Lettinga *et al.*, 1980), fluidized bed (Cooper & Sutton, 1983) and expanded granular sludge blanket (Frankin *et al.*, 1992).

The aim of this study was to treat AMD with synthetic domestic wastewater sludge and to investigate the microbial ecology of the process using different variables.

2. Materials and methods

2.1 The treatment of AMD with synthetic anaerobic domestic wastewater sludge in sterile bioreactors for 90 d

Synthetic anaerobic domestic wastewater sludge (SDWWS) was prepared as described in Chapter 3 (Table 1). Medical drip bags (1 L) (Stelmed, Stellenbosch, South Africa) served as miniature bioreactors (Figure 1). AMD sampled from the Exxaro Coal mine was couriered overnight in 5 L plastic containers and stored at room temperature (20-21°C) until use. AMD and SDWWS media were added to sterile saline medical drip bags (Stelmed) in a 1:1 ratio to a final volume of 900 mL in triplicate. Two different control bioreactors were included in triplicate: one control bioreactor consisting out of 1:1 dH_2O to AMD mixture and a medium control bioreactor consisting out of a 1:1 SDWWS: dH_2O mixture. Anaerobic domestic wastewater sludge, collected from the anaerobic digester tank at the Pniel wastewater treatment plant, served as an inoculum and 10 mL was added to the three trial bioreactors respectively. The three trial bioreactors and the control bioreactors were incubated at 25 °C in a dimly lit environment for a period of 90 days. Samples (5 mL) of the liquid contents from each bioreactor were extracted before incubation and after 90 days by using a sterile syringe and needle. The chemical oxygen demand (COD) and sulphate concentration of each extracted sample were measured using the Merck Spectroquant Pharo 300 and cell test kits according to the respective protocols. The pH was determined by using a digital pH meter (PCTestr 35 Multi-Parameter). The ambient temperature was monitored twice daily with a

hand held thermometer (Pocket Thermometer MT605, Allen Instruments, Cape South Africa). The trial was conducted in duplicate.

Table 1. Composition of the media used as synthetic anaerobic domestic wastewater sludge.

Component	**Mass mg/L**
Meat extract	2182
Vegetable extract	218
NaCl	72.7
$MgSO_4$	182
KH_2PO_4	145
$FeSO_4$	36
Glucose	182

Figure 1. The control bioreactor (far left) and three trial bioreactors (the remaining three bioreactors) containing AMD and SDWWS medium in a 1:1 ratio on Day 0 of incubation.

2.2 The investigation of the treatment of AMD with synthetic anaerobic domestic wastewater sludge in sterile bioreactors for a reduced incubation time.

To determine if the COD and the sulphate concentration can be reduced during the co-treatment of AMD and SDWWS in a shorter treatment time, the above described experiment was repeated. The COD and sulphate concentrations were measured before incubation and during the trial as described above until sulphate reduction reached a plateau. The pH was measured at the initiation of the trial and the pH adjusted to 7.5 if needed. The ambient temperature was monitored twice daily with a hand held thermometer (Pocket Thermometer MT605, Allen Instruments, Cape South Africa).

2.3 The treatment of AMD with SDWWS in bioreactors with an established biofilm for a reduced incubation time.

Three 1:1 AMD to SDWWS bioreactors were prepared and incubated as previously described for a period of 30 d in a dimly lit environment where after the liquid content of the bioreactors was discarded and replaced with fresh AMD and SDWWS media in a 1:1 ratio. The bioreactors were incubated as previously described for a period of 30 d. The COD and sulphate concentration were measured before incubation and regularly thereafter as describe before. The ambient temperature was monitored twice daily with a hand held thermometer (Pocket Thermometer MT605, Allen Instruments, Cape South Africa). Again two control bioreactors were included: one control bioreactor consisting out of 1:1 dH_2O to AMD mixture and a medium control consisting out of a 1:1 SDWWS: dH_2O mixture.

2.4 The effect of reduced incubation temperature on the treatment of AMD with SDWWS in sterile bioreactors

A set of two control bioreactors and three 1:1 AMD to SDWWS bioreactors were prepared as described previously and incubated at 17-19°C in a dimly lit environment. The COD and sulphate concentration were monitored before incubation and during the trial until a plateau was reached. The ambient temperature was monitored twice daily with a hand held thermometer (Pocket Thermometer MT605, Allen Instruments, Cape Town, South Africa).

2.5 Chemical analyses of precipitants formed in the bioreactors

A white precipitant formed at the top of the bioreactors during the trials that were incubated at a reduced incubation time and bioreactors with an established biofilm. A bioreactor from the reduced incubation time trial was emptied and the white substance scraped off with a sterile blade. Thereafter the sampled precipitant was inserted into a sterile Eppindorf tube and sent to the Council for Scientific and Industrial Research (CSIR) in Stellenbosch for analyses using the ICP OES method.

2.6 Determining the microbial diversity present in the bioreactors after the AMD treatment trial period

2.6.1. Enumeration of total anaerobic bacteria in bioreactors

SDWWS media (500 mL) was prepared as described before and 2 % Agar (Sigma-Aldrich) was added to prepare agar growth plates. The bioreactors of the 90 d pioneer trials, the reduced incubation time trial and the incubation time biofilm trial were lightly mixed and 1 mL of the liquid content was sampled where after serial dilutions up to 10^{-4} were prepared in sterile distilled water. Nine hundred microlitres of sterile dH_2O was used for the serial dilution with an inoculum of 100 µL. Subsequently 100 µL of the dilution series was spread plated onto the agar growth plates as described above. The agar plates were incubated under anaerobic conditions at 25°C using an airtight plastic container and an anaerobe pack (Davis Diagnostics) for a period of 7 days.

The bacterial inhabitants of the reduced incubation temperature trials were not enumerated as the mentioned trials failed to perform in terms of sulphate and COD removal.

2.6.2 Visualisation of the microorganisms present in the bioreactors through Transmission electron microscopy

A sterile syringe and needle were used to extract 10 mL of one of the reduced incubation temperature trials and one of each of the 90 d trial bioreactors. The samples were centrifuged at 6 000 rpm for 2 min (the standard centrifugation operation for the duration of this section) where after the resulting pellet was resuspended in 2.5 % glutaraldehyde (Agar Scientific) in fixation buffer (0.1M sodium cacodylate (Agar Scientific), 2mM MgCl, pH 7.4) at 4°C overnight. The samples were again centrifuged and rinsed twice with fixation buffer. A post fixation for 1 h in 1 % osmium tetroxide (Electron Microscope Science) at room temperature (21°C) took place followed by another centrifugation and triple wash steps with distilled water. The samples were stained with En-bloc stain for 30 min with 2 % uranyl acetate (Agar Scientific). The samples were again centrifuged and the pellets washed with distilled water and the wash step repeated. The samples were spun down and resuspended in the following ethanol (Merck) concentrations respectively for 5 min in each concentration: 30 %, 50 %, 70 %, 80 %, 90 % and 95 %. Thereafter followed a 100 % ethanol exposure step where the samples were again spun down and resuspended in 100 % ethanol for 10 min which was repeated and followed by two 100 % acetone (Merck) steps, also for the duration of 10 min.

The resin infiltration was conducted as follows: The samples were resuspended in 50 % resin (Agar Low Viscosity Resin) (diluted with acetone) overnight, thereafter resuspended in 75 % resin for 6 h, followed by a re-suspension in 100 % resin overnight and again 100 % resin for 1 h. The samples were embedded in molds and baked at 60°C for 24 h and cut into thin sections using an ultramicrotome (Reichert Ultracut S, Leica Microsystems, Vienna, Austria) The resulting sections were stained with lead citrate and visualised using the transmission electron microscope (TEM) (FEI Tencai 20 transmission electron microscope, FEI, Eindhoven, Netherlands) operating at 200 kV (Lab6 emitter) and fitted with a Tridiem energy filter and Gatan CCD camera.

2.6.3 PCR and next generation sequencing (Ion Torrent)

One bioreactor from each mentioned trial describe before, was well shaken and a sample was extracted before and after incubation using a sterile needle and syringe. A total of 4 mL from the respective samples were centrifuged where after the resulting pellets were re-suspended in 200 mL dH_20. For total genomic DNA extraction from each sample, the ZR^{TM} Soil microbe DNA Miniprep Kit (Inqaba Biotech, Johannesburg, South Africa) was used. Genomic DNA was isolated according to the manufacturer's instructions. The resulting gDNA was sent to the Central Analytical Facility (CAF) of the PCR and for next generation sequencing (NGS). The variable region 3 and 4 (V3-V4) of the 16S rRNA genes of the bacteria were amplified from gDNA using fusion primers (IDT, US) (Table 2). Library templating and amplification was performed using the Ion OneTouch 2 instrument (Lifetech). Sequencing was performed using the Ion Torrent PGM system using the Ion 318 chip version 2 (Lifetech). Initial amplification of the gDNA samples was not successful due to potential PCR inhibitors present in the samples. Therefore the gDNA was again extracted and pre-treated with activated carbon, as described below, in order to remove PCR inhibitors (Abolmaaty *et al.*, 2007).

2.6.3.1 Sample pre-treatment with activated carbon

Samples were extracted from the bioreactors before and after incubation as described above. A total of 4 mL from the respective samples were centrifuged where after the resulting pellets were resuspended in 200 µL dH_20. The resulting 200 µL solution was exposed to a 2 mm layer of activated carbon (Jacobi Carbons, Germany) for a range of periods (30 min, 60 min, 90 min and 2 h) at room temperature (23°C) in order to find the minimal time of exposure required to reduce the amount of PCR inhibitors sufficiently to allow polymerase chain reaction (PCR) (Abolmaaty *et al.*, 2007). The samples were centrifuged and the resulting

pellets resuspended in 200 µL dH_20. A ZR™ Soil microbe DNA Miniprep Kit (Inqaba Biotech, Johannesburg, South Africa) was then used and the recommended protocol followed. The resulting gDNA was sent to the Central Analytical Facility (CAF) of the PCR and next generation sequencing (NGS) process. The 16S rRNA genes of the bacteria were amplified from gDNA using Fusion primers (IDT, US) using an Ion Torrent PGM sequencer (Lifetech). Barcodes were used to allow multiplexing during the Ion Torrent sequencing processing (Ion Express barcodes, IDT).

The sequencing data obtained was analysed using Mothur SOP 454 software according to the recommended protocol (Schloss *et al.*, 2011). The sequence of the Fusion primer was 5'-ACTCCTACGGGAGGC-3'.

3. Results and discussion

3.1 The treatment of AMD with SDWWS in sterile bioreactors for 90 d.

The average reduction of COD within 90 d in the bioreactors was 86.8 % and 85 % respectively (Table 2) and the sulphate concentration was reduced by 98.73 % and 99.12 % (Table 3). The AMD:dH_2O control bioreactor was able to remove 0.83 % of the COD content and showed an increase of 2 % in sulphate content. The COD and sulphate levels of the SDWWS: dH_2O controls remained stable except for an increase of 4.15 % in the sulphate levels of the 2nd trial. The increase in sulphate in the control might be due to the release of sulphate from sulphate bound compounds. The liquid contents in the bioreactors also developed a bright green colour after the third day in the 90 d trial and remained so until termination (Figure 2).

Table 2. COD reduction in AMD after 90 d treatment with SDWWS.

		Day 1	**Day 90**	**Reduction (%)**	**Std Dev**
1st Trial	Control (AMD:dH_2O)	196.00	190.00	3.06	4.1
	Medium control	1500	1504	-0.26*	2.7
	Ratio 2	1530.00	202.00	86.76	5.1
2nd Trial	Control (AMD:dH_2O)	480	476	0.833	3.5
	Medium control	1498	1510	-0.8*	6.5
	Ratio 2	2090	252	85	7.1

*The negative value indicates an increase in COD concentration.

Table 3. Sulphate reduction in AMD after 90 d treatment with SDWWS.

		Day 1	Day 90	Reduction (%)	Std Dev
1st Trial	Control (AMD:dH_2O)	490	470	4.08	6.1
	Control (SDWWS:dH_2O)	300	295	1.50	4.3
	Ratio 2	550	7	98.73	4.9
2nd Trial	Control (AMD:dH_2O)	490	500	-2.04*	1.8
	Medium control	289	301	-4.15*	3.2
	Ratio 2	493.33	4	99.19	4.9

*The negative value indicates an increase in sulphate concentration.

Figure 2. Digital images showing the colour change of the liquid contents in the bioreactors during the 90 d AMD treatment trial. a) The control (left in image a) and the bioreactor after the 90 d AMD treatment trial (right & b).

3.2 The treatment of AMD with SDWWS in sterile bioreactors for a reduced incubation time.

The bioreactors incubated at a reduced incubation time showed an average reduction in COD (Figure 3) and sulphate (Figure 4) levels of 60.8 % and 96 % respectively after 26 d after which a plateau was reached for both in the 30 d treatment trial. From here on this trial will be referred to as the '30 d pioneer trial'. The AMD: dH_2O control showed 61 % increase in COD and virtually no change in sulphate levels whereas the SDWWS: dH_2O control had virtually no change in either COD or sulphate levels. The liquid contents in the bioreactors changed from transparent to dark brown during the 30 day incubation period and a white precipitant

was seen at the top of the bioreactors (Figure 5). A possible reason why the COD decreased only by 60.8 % in comparison to the 86 % reduction in the 90 d trial, is that sampling took place in different seasons. Temperature changes and rainfall could impact the microbial community present in the anaerobic digester tank from which the inoculum was taken.

A study conducted by Hughes and Gray (2013) delivered similar findings. Hughes and Gray (2013) investigated the co-treatment of anaerobic domestic wastewater sludge and AMD with regards to COD, sulphate and heavy metal removal. Synthetic AMD was used in combination with different domestic wastewater sludge and municipal wastewater samples in bench scale sequencing batch reactors operating at 20°C ± 2. Co-treatment of AMD and domestic wastewater sludge had limited ability to reduce COD. Also, a netto COD decrease of about 50 % was archived. The exhaustion of sulphate has been eliminated as the bioreactor in above mentioned study was dosed with AMD after the COD decrease plateau was reached and showed no impact on the reduction of AMD. A possible explanation could include a change in metal species as heavy metals showed to influence the availability of COD (Giesy & Briese, 1977; McKnight *et al.*, 1992). As the treatment progresses, SRB removed heavy metals during AMD treatment operations (McCauley *et al.*, 2009). Another possibility is the microbial community present as the microbial community of every bioreactor is unique. Vieira and co-workers (2014) treated synthetic AMD in a 6 L bench-scale reactor at 30°C with ethanol as an energy source and used sludge obtained from an up-flow anaerobic sludge bed reactor treating slaughterhouse waste as an inoculum. Sulphate and COD reduction were 10 % and 53 % respectively at an initial pH of 7 and increased to 42 % and 99 % at an initial pH of 4. These processes occurred within 10 h. Although Vieira and co-workers achieved a greater sulphate reduction after a shorter treatment time the uneconomical implications of the treatment at 30°C and the use of ethanol as a carbon source should be kept in mind as external temperatures varies and heating of the bioreactor may become expensive and not viable.

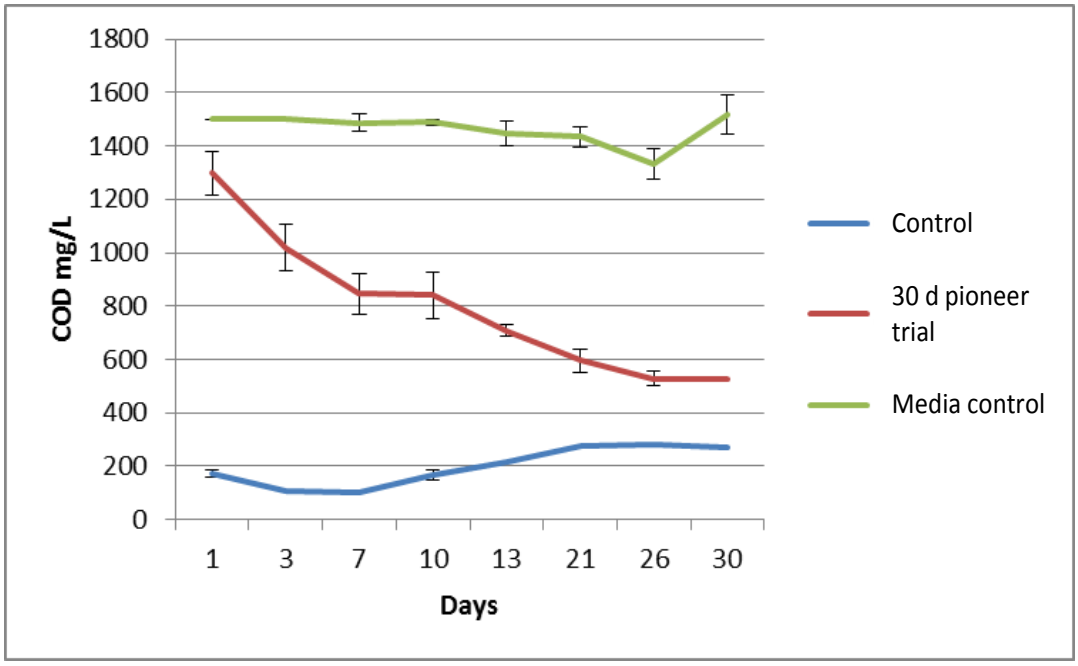

Figure 3. COD reduction in AMD treated with SDWWS in the 30 d pioneer trial.

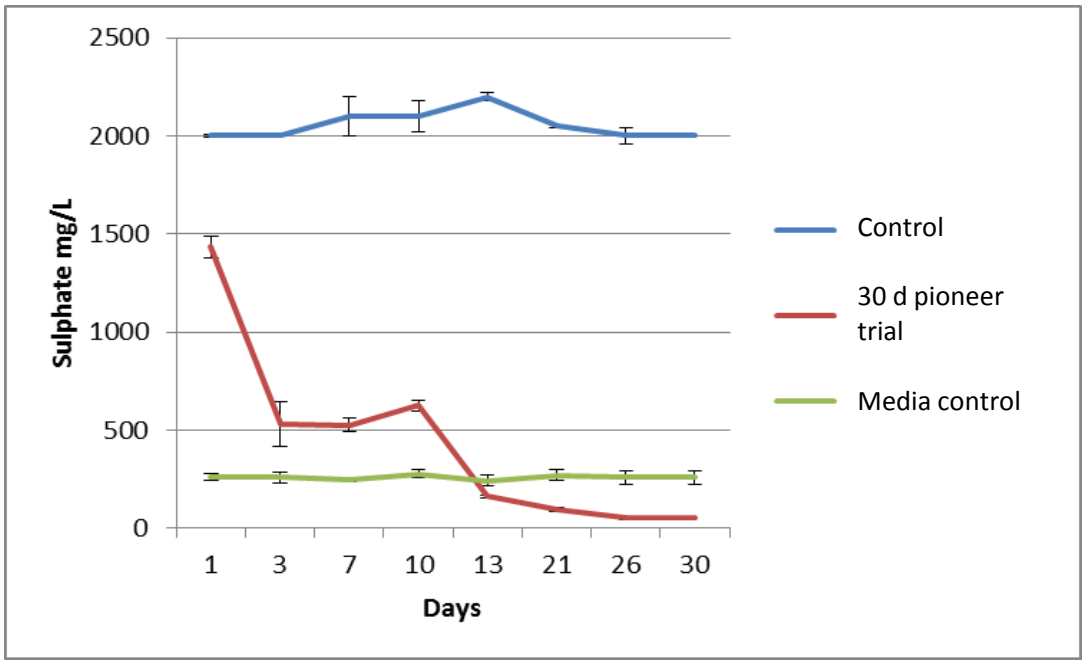

Figure 4. Reduction of sulphate concentration in AMD treated with SDWWS during the 30 d pioneer trial.

Figure 5. Digital image showing white precipitation formed in the bioreactors and the dark brown colour of the contents. The control bioreactor (far left) and triplicate bioreactors at the time of termination of the 30 d pioneer trial.

3.3 The treatment of AMD with SDWWS in bioreactors with an established biofilm for a reduced incubation time.

In the bioreactors with an established biofilm, the COD and sulphate levels reduced by an average of 58 % (Figure 6) and 96 % (Figure 7) respectively and were therefore similar to the results of the 30 d pioneer trial. This is contradictory to the hypothesis that the presence of a biofilm in the bioreactors will result in higher COD and sulphate removal. COD and sulphate removal in wastewater that has been in contact with biofilms, were reported (Lazarova & Manem, 1995; Nicolella *et al.,* 2000; Wuertz *et al.,* 2003). A possible explanation for the results in this study is that only a small percentage of the liquid content in the bioreactors was in contact with the biofilm. There was also no visible difference in the colour of the liquid contents between the 30 d pioneer trial (Figure 5) and this trial (Figure 8). Both the AMD:dH_2O control and the SDWWS:dH_2O control showed virtually no change in either the sulphate or the COD levels. From here on this trial will be referred to as the '30 d biofilm trial'.

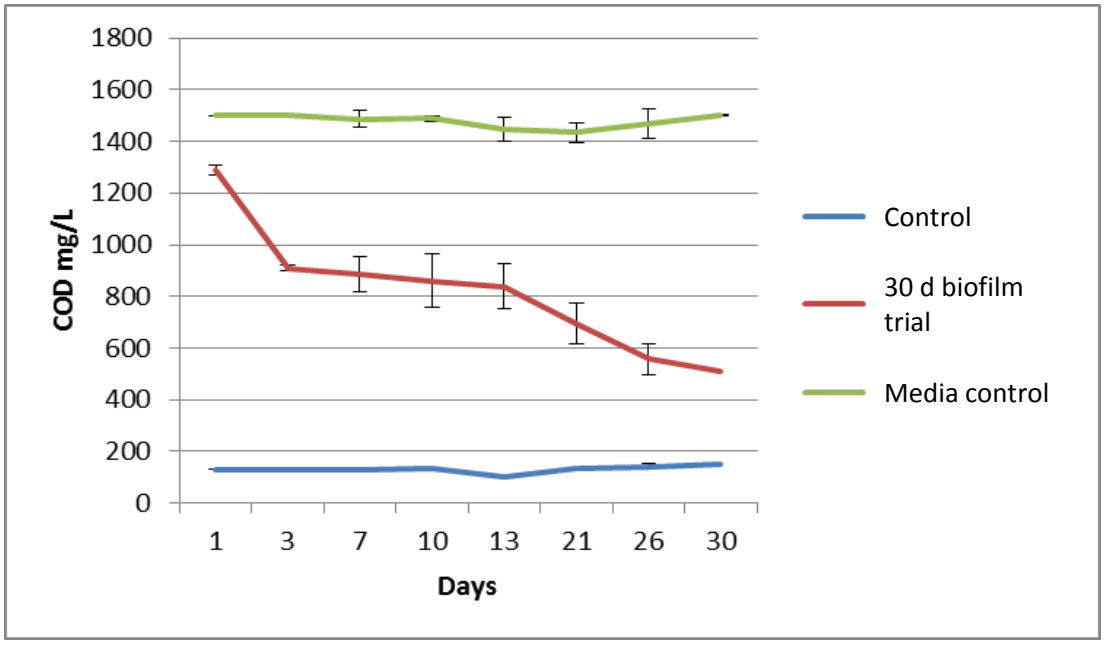

Figure 6. COD reduction in AMD treated with SDWWS in the 30 d biofilm trial

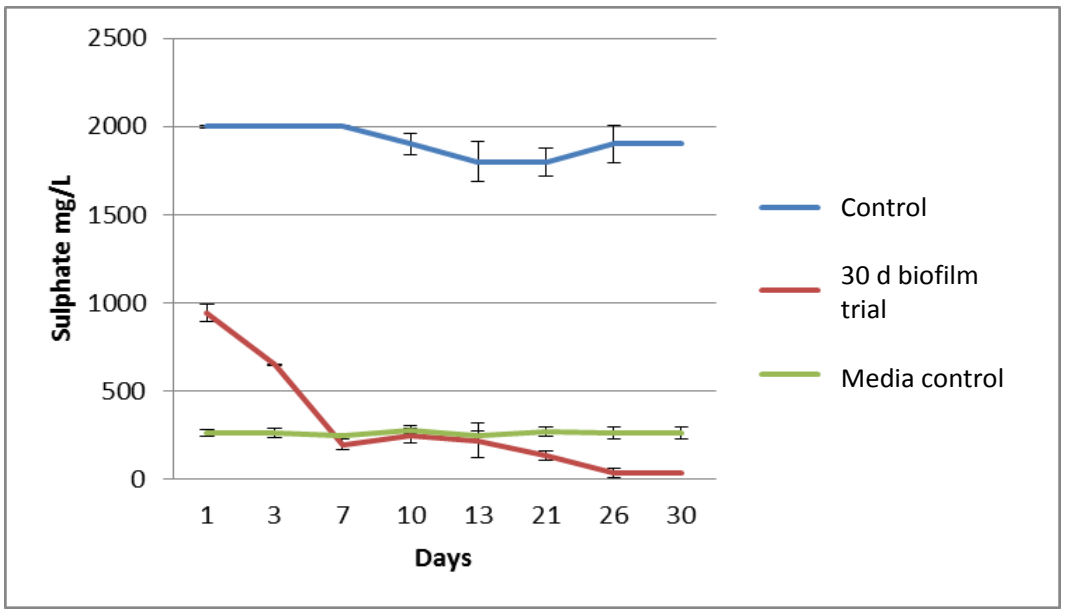

Figure 7. Reduction of sulphate concentration in AMD treated with SDWWS during the 30 d biofilm trial.

Figure 8. Digital image showing white precipitation formed in the bioreactors and the dark brown colour of the contents. The control bioreactor (far left) and triplicate bioreactors at the time of termination in the 30 d biofilm trial.

3.4 The effect of reduced incubation temperature on the treatment of AMD with SDWWS in sterile bioreactors.

The trial conducted at 17-19°C resulted in an average COD reduction of 12 % (Figure 9) and 10 % reduction in sulphate concentration and reached a plateau after 20 d (Figure 10). Virtually no change in COD and sulphate concentrations were found in the SDWWS:dH_2O control. However, the AMD:dH_2O control showed a 20 % decrease in COD and stable sulphate levels. Therefore when comparing the results to the trials conducted at 25°C, it is clear the treatment requires higher temperatures. A study conducted by Poinapen and co-workers supports the importance of incubation temperature (Poinapen *et al.*, unpublished date). They investigated the treatment of AMD regarding sulphate reduction using primary domestic sewage sludge in an upflow anaerobic sludge bed reactor at 20ºC and 35 ºC respectively. They found a delayed start-up and a reduced COD removal in the 20ºC reactor.

Greben *et al.* (2002) also emphasized the importance of incubation temperature and the reduced sulphate reduction rate under conditions of lower temperatures. Greben and co-workers used anaerobic batch bioreactors to test the effect of lower temperatures and carbon source on the rate of sulphate reduction. Synthetic AMD and a mixture of technical grade ethanol as carbon source were used. At ≤15˚C practically no sulphate reduction took place whereas a bioreactor operating at 20˚C was able to remove 7.80 g sulphate/d.

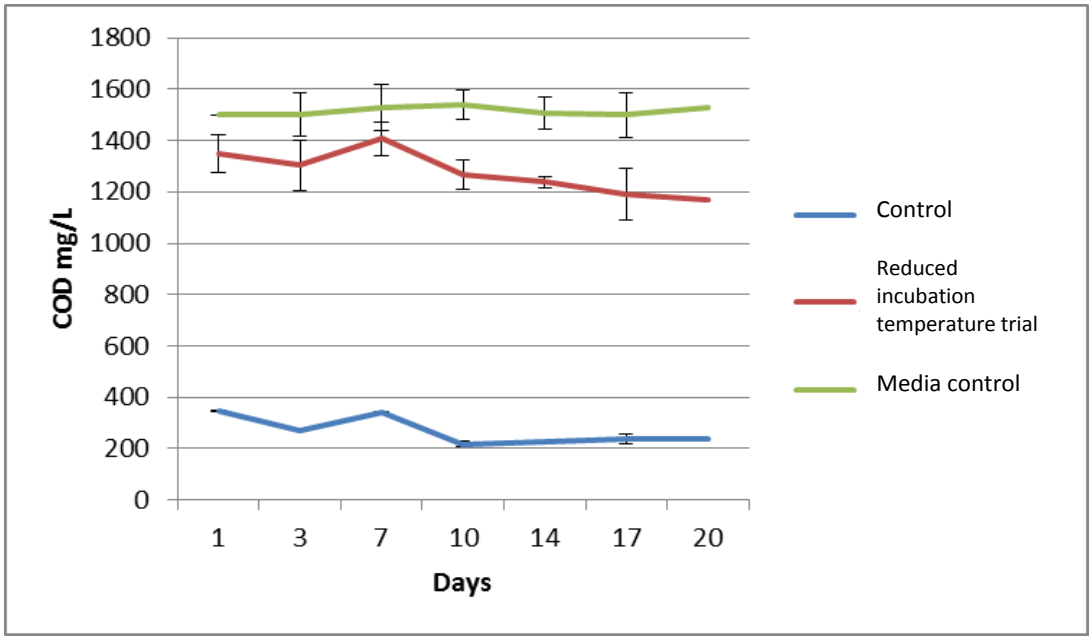

Figure 9. The effect of reduced incubation temperature on COD reduction during the treatment of AMD with SDWWS in sterile bioreactors.

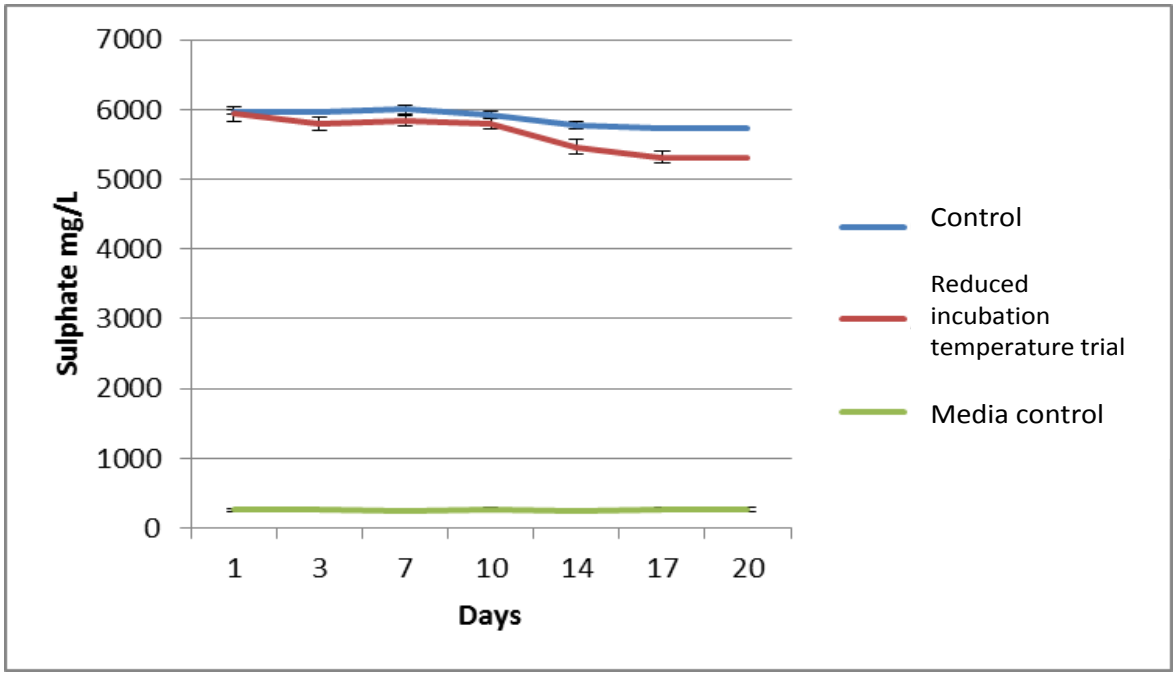

Figure 10. The effect of reduced incubation temperature on the reduction of sulphate concentrations during the treatment of AMD with SDWWS in sterile bioreactors.

3.5 Chemical analyses of precipitants formed in the bioreactors

A white substance at the top of the bioreactors from the 30 d pioneer and biofilm trials formed (Figures 5 & 8) and was analysed using the ICP OES method (Table 4) to determine the level of potassium, sodium, calcium, magnesium, sulphate and total organic carbon present. The main components of the precipitated substance were sulphate (207 mg/L) and organic carbon (229 mg/L). This indicated the possible presence of *Chlorobium* spp. in the bioreactors as these species precipitate sulphur in the form of globules (Pott & Dahl, 1998).

Table 4. The ICP OES analyses of the white precipitation formed in the bioreactors during the trials.

Analysis	mg/L
Potassium as K Dissolved	32
Sodium as Na Dissolved	14
Calcium as CA Dissolved	36
Magnesium as Mg Dissolved	1.6
Sulphate as SO4 dissolved	207
Total Organic Carbon	229

3.6 Determining the microbial diversity present in the bioreactors after the AMD treatment trial period

3.6.1 Enumeration of total anaerobic bacteria in bioreactors

The total anaerobic bacteria from the 90 d pioneer bioreactor trial obtained by spread plating onto agar plates containing SDWWS was 1.08 x 10^5 and 7 x 10^4 CFU/mL for the control (Table 5). The colony forming units on the agar plates were visually inspected and no difference was observed regarding microbial diversity between the control and the trial bioreactor plates.

The plate counts after the 30 d pioneer trial was 1 x 10^5 and 6.5 x 10^4 in the control bioreactor (Table 5). The plate counts obtained from the 30 d biofilm trial was 1.32 x 10^5 and 1.29 a 10^5 in the control bioreactor. The high plate count in the control can be attributed to the established microbial population in the biofilm.

Table 5. The average plate counts obtained by the various trials.

Trial	**Average count (CFU/mL)**
Control 90 d pioneer trial	7×10^4
90 d reactor (average)	1.08×10^5
Control 30 d pioneer trial	6.5×10^4
30 d pioneer trial (average)	1×10^5
Control 30 d biofilm trial	1.29×10^5
30 d biofilm trial (average)	1.32×10^5

3.6.2 Visualisation of the microorganisms present in the bioreactors through transmission electron microscopy

To visualise and compare the contents of two trials where COD and sulphates were reduced by a low percentage and a high percentage respectively, the contents of the bioreactors incubated at a reduced temperature (Figure 11) and the bioreactors from the 90 d pioneer trial were visualised through TEM (Figure 12). The images taken from the reduced incubation temperature trials revealed black precipitate that might be iron sulphide. Few micro-organisms were observed when compared to the images of the 90 d pioneer trials. However, no conclusions can be made from this observation as the TEM technique is qualitative and not quantitative. The images obtained do not represent the bioreactor as a whole.

TEM images obtained by the 90 d pioneer trials revealed the images of what possibly are green sulphur bacteria. Indicated with arrows in the respective images in Figure 12 are potential sulphur globules, characteristic of *Chlorobium* spp.

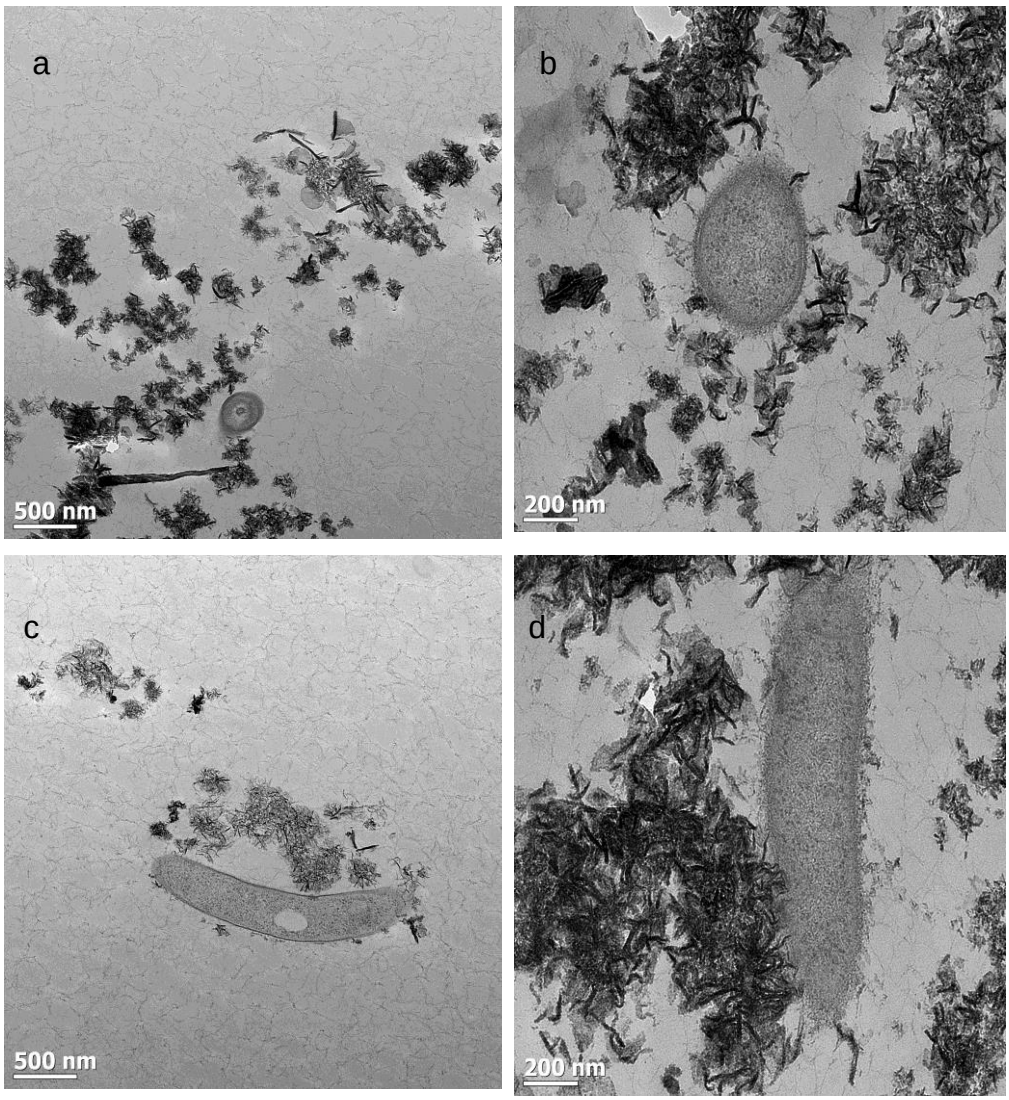

Figure 11. a & c) Overview images of the reduced incubation temperatures trials at 500 nm. b & d) Images of the reduced incubation temperature trials at 200 nm.

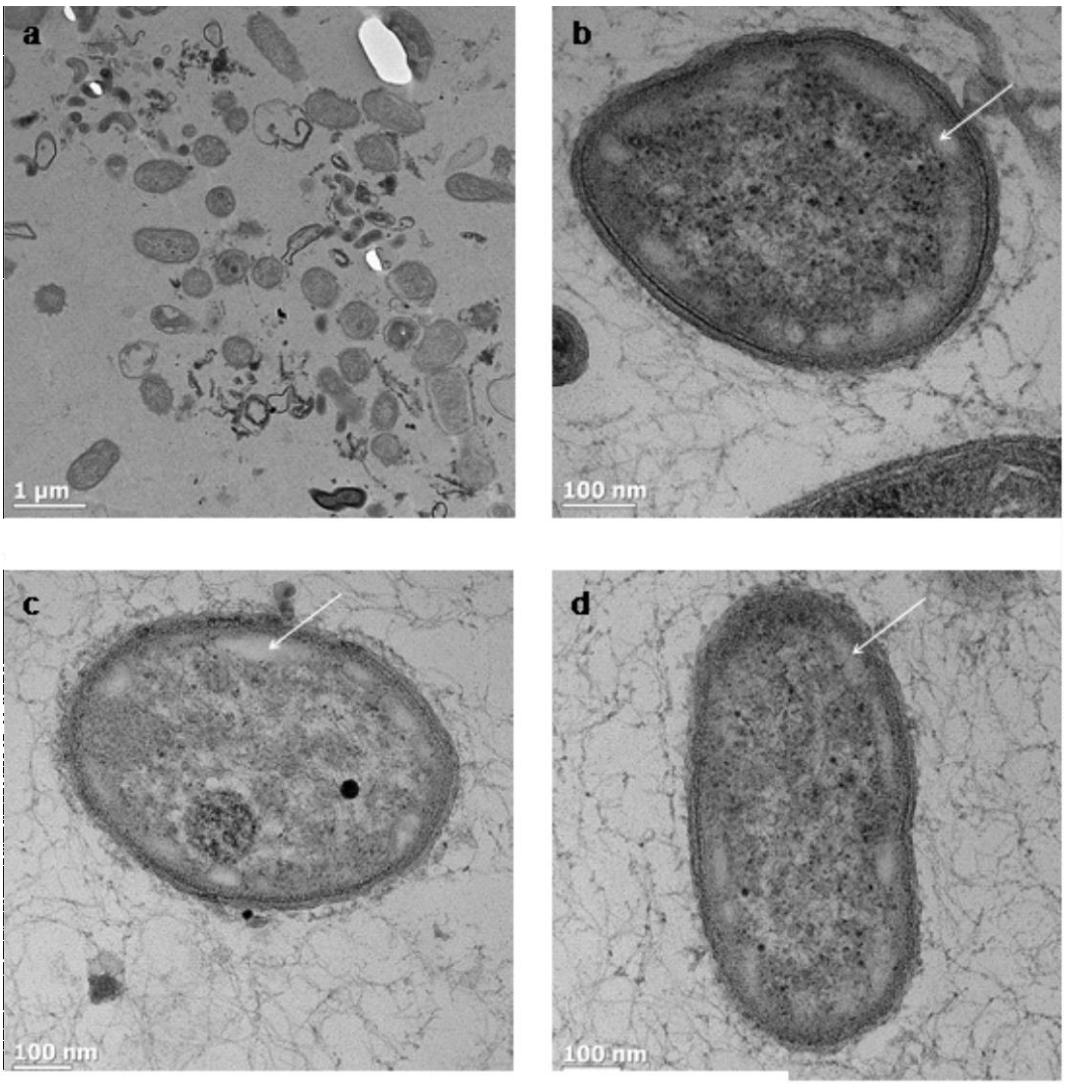

Figure 12 a) The TEM images obtained by the 90 d pioneer trials on a 1 µm scale. b-d) TEM images obtained by the 90 d pioneer trials on a 100 nm scale. The arrows indicate potential sulphur globules.

3.6.3 PCR and Next generation sequencing (Ion Torrent)

3.6.3.1 Sample pre-treatment with activated carbon

The 2 h period of activated carbon exposure proved to be sufficient as pre-treatment for DNA extraction for all the samples. The required exposure time needed for the activated carbon to reduce the PCR inhibitors sufficiently in order to allow the PCR process was 4 times more than recorded in literature as Abolmaaty and co-workers exposed oyster samples to activated charcoal for a period of 15 min (Abolmaaty *et al.*, 2007). This may be caused by a larger amount of PCR inhibitors present in the current study's gDNA samples than that of Abolmaaty *et al.* (2007).

3.6.3.2 Comparison of the dominant microbial species present in the beginning and end of each trial

Data produced by next generation sequencing from each trial before and after treatment was compared (Figure 13). The main contributors to the microbial composition of the respective trials at the beginning and end of the respective duration periods, in terms of numbers of sequences of each micro-organism obtained compared to the total number of sequences obtained by the respective trials, were expressed as a relative percentage.

With regards to the interpretation of the sequencing data obtained it should be stressed that the number of sequences obtained from each trial is not necessarily reflective of the number of microbial organisms present in the particular bioreactor as the gDNA obtained was not quantified. From this point forward this will be referred to as a relative number or a relative percentage.

A dominance of *Chlorobium* spp. was detected at the end of all the trials except in the first reduced incubation temperature trial. This finding supports the sulphate precipitant found in the bioreactors (Table 5). The importance of incubation temperature on the selection of bacteria has been stated (Madigan *et al.,* 1997). The highest amount of *Magnetospirillum* spp. was present in the second bioreactor of the reduced incubation temperature trial (20 %) in comparison to the first bioreactor from this trial (0 %) (Figure 13). This was not unexpected as some *Magnetospirillum* spp .are strict anaerobes and use iron as an electron donor (Zhu *et al.* 2010). This supports the general characteristic of AMD being high in heavy metal concentration (Geremias *et al.,* 2003). The *Magnetospirillum* spp. numbers in the 30 d pioneer trial (4 %) was higher than in the 30 d biofilm trial (1.2 %). The environment created by the 90 d pioneer trials possibly selected against *Magnetospirillum* spp. Another possibility is competition between *Magnetospirillum* spp. and the dominant bacterial genera, *Chlorobium* spp. (Hibbings *et al.,* 2009). *Clostridium* spp. was detected in all trials but the highest percentage was found in the two 90 d pioneer trials with 5.2 % and 8.8 % respectively. Again this could be attributed to the inoculation amount as the samples taken at the beginning of the 90 d pioneer trials revealed a *Clostridium* spp. count (20 %) much higher than the other trials that varied between 13 % and 0.1 %. *Clostridium* spp. has also been detected in wastewater treatment processes by Burns and co-workers (2012).

The percentage *Turneriella* spp. found in the 90 d pioneer trials was 1.5 % and 2.9 % respectively, the reduced incubation temperature trials 0 % and 0.8 % respectively, the 30 d pioneer trial 1.3 % and the 30 d biofilm trial 1 %. The amount of *Turneriella* spp. detected at

the beginning of each trial reflected the amount at the end as the amounts varied between 0.6 % and 1.1 %. *Desulfovirga* spp. was present at the beginning of the 90 d pioneer trial at a relative 16 % however there was little trace of it at the end of these trials (1 % and 0.58 % respectively). In the case of the other trials the relative starter percentages varied between 0 % and 0.1 % and the relative end percentages were 0 % and 0.5 %. *Pseudomonas* spp. was present at the beginning of all trials, at 1.18 % in the case of the 90 d pioneer trials, 2.25 % in the case of the reduced incubation temperature trials, 3.43 % at the beginning of the 30 d pioneer trial and 9.09 % in the case of the 30 d biofilm trial but were undetected at the end of all the respective trials, except for the second bioreactor of the reduced incubation temperature trials (7.24 %) and the 30 d biofilm trial (0.38 %). *Azospirillum* spp. was detected in the second bioreactor of the reduced incubation temperature trial (13 %) and at the beginning of the 30 d pioneer trial (4.7 %) despite *Azospirillum* spp. not being detected at the beginning of the reduced incubation temperature trial. This emphasizes the importance of perspective when using next generation sequencing and interpreting the results as it is possible that *Azospirillum* spp. was present at the beginning of the reduced incubation temperature trial but was not detected.

Gordonia spp. was present in the first bioreactor of the reduced incubation temperature trial after 20 d at 1.67 %, but was not detected at the beginning of the trial. However, *Gordonia* spp. was detected at the beginning of the remainder trials at 0.44 % in the case of the 90 d pioneer trials, 2.29 % in the 30 d pioneer trial and 0.88 % in the case of the 30 d biofilm trial. *Elizabethkingia* spp. was detected in the beginning of the 90 d pioneer trial (0.59 %), at the beginning of the reduced incubation temperature trial (0.31 %), in the sample from the second bioreactor from the reduced incubation temperature trial (7.84 %) and at the beginning of the 30 d biofilm trial (1.73 %). In the remainder of the trials species from this genus were undetected. In the case of all but the second bioreactor from the reduced incubation temperature trial *Haliscomenobacter* spp. was undetected (at a relative percentage of 0.5 %) except for being detected at the beginning of the 90 d pioneer trials (0.15 %) and the beginning of the 30 d pioneer trial (5.86 %). *Sulfurospirillum* spp. was present at a relative amount of 1.78 % at the beginning of the 90 d pioneer trials but was detected at a relative amount of 0.12 % in the second 90 d pioneer trial only. At the beginning of the reduced incubation temperature trial *Sulfurospirillum* spp. was detected at 1.78 % in contrast to the 1.88 % at the end of the 1st trial. *Sulfurospirillum* spp. was not detected at the end of the 2nd reduced incubation temperature trial. At the beginning of the 30 d pioneer trial *Sulfurospirillum* spp. was undetected but at the end of this trial was detected at a relative

amount of 0.57 %. Similarly, *Sulfurospirillum* spp. was detected at a relative percentage of 0.19 % at the end of the 30 d biofilm trial but was undetected at the beginning of the trial. This put further emphasis on the importance of perspective when dealing with next generation sequencing technology (Shendure & Ji, 2008).

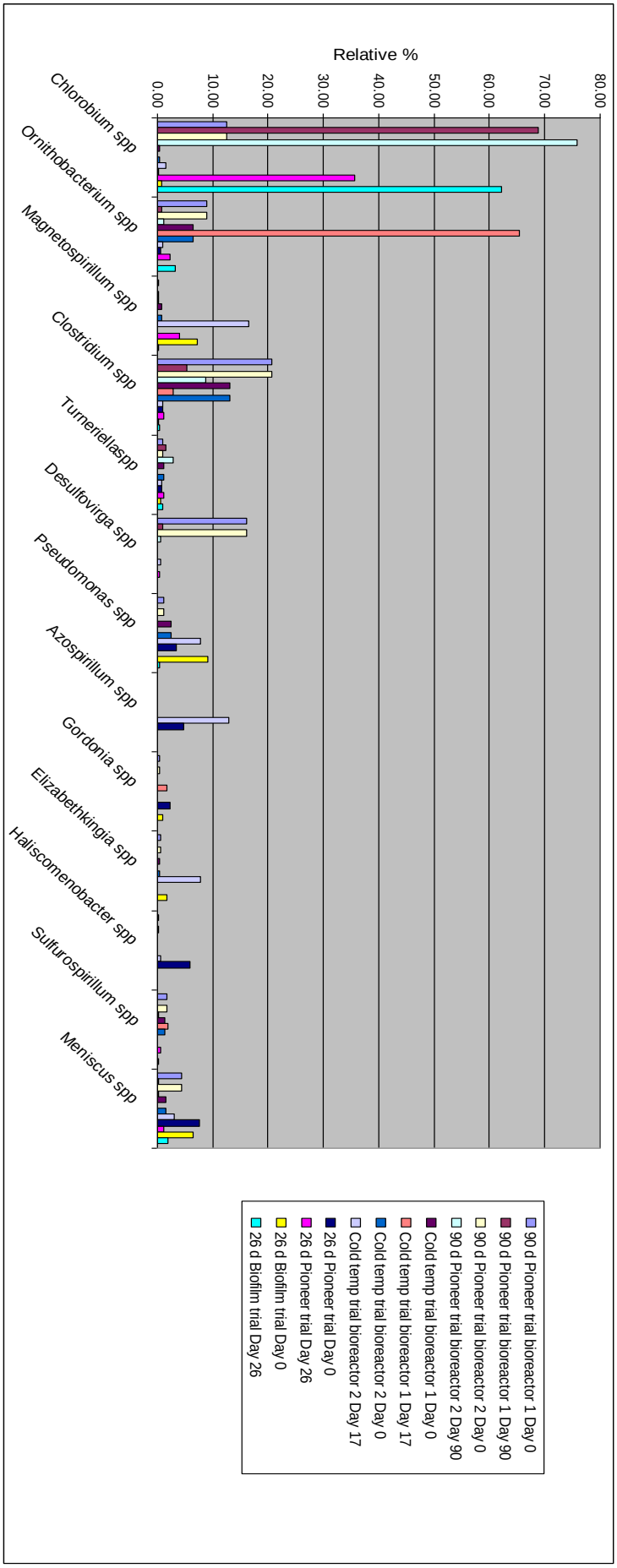

Figure 13. A summary of the top 50 microbial genera (otu's) found during this study and the abundance of these microbes in the respective trials (see Addendum A for the respective genera represented by each out).

3.6.4. Description of sequence analyses for each trial

3.6.4.1. Sequence analyses of the 90 d pioneer trials

Despite some variation between the two bioreactors from the 90 d pioneer trials the following section will treat the two bioreactors as a whole for the discussion of possible trend commencing in such a bioreactor under the relevant environmental circumstances. There was very little variation between the microbial makeups of the trials on the day of trial initialization (indicated in all the graphs to follow as Day 0).

Species from the genera *Chlorobium* spp. dominated the 90 d pioneer at the end of the treatment process by 68 % (first trial) and 76 % (second trial). *Chlorobium* spp. is a genus of green sulphur bacteria that contain bacteriochlorophyll as a light harvesting pigment and explains the green colour of the liquid contents of the bioreactors (Figueras *et al.*, 1997) (Figure 2). The findings of Hesham & Yang (2011) support the dominant presence of *Chlorobium* spp. found in this study. Sulphate reducers (*Desulfovirga* spp.) were detected at low relative levels. These results coincide with the findings of phototrophic bacterial wastewater treatment systems being an alternative to conventional treatment options (Almasi & Pescod, 1996). A low number of *Flavobacterium* spp. relative to the number of *Chlorobium* spp. was detected. As many phototrophic bacteria are known to produce iron-sulphur proteins (Renger, 2008) some of the available sulphur would likely be utilized for the production of such proteins, thereby removing the involved sulphur from the environment.

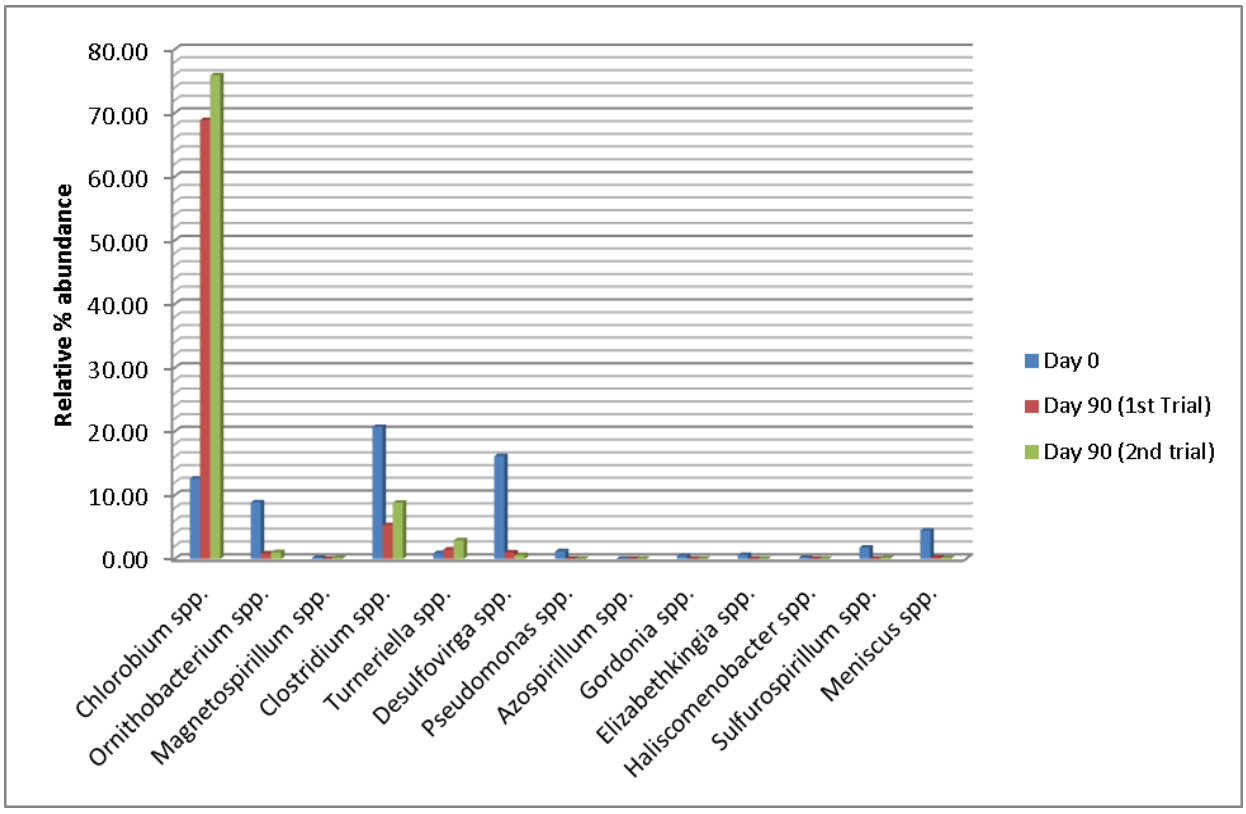

Figure 14. The microbial data obtained from the 90 d pioneer trials.

Turneriela spp. is an aerobic spirochete that prefers tropical climate (Centre for disease Control, 2003). A possible explanation for the presence of *Turneriela* spp. in the anaerobic conditions of the bioreactors is that it grew in numbers after the initialization of the trials and halted growth or died off some time thereafter. It should be taken into account that it is not possible to tell distinguish dead cells from living cells via DNA sequencing as dead and alive cells are disrupted when extracting the gDNA.

A conceptional model regarding the sulphur cycle in the mentioned trials was proposed (Figure 15) (Table 6). The netto reactions (indicated in a bold arrow) shows the metabolic conversion of sulfate and hydrogen sulfide to elemental sulphur which likely is deposited in globules by *Chlorobium* spp. and therefore removed from the system. The other main reaction is the utilization of sulphur for the production of iron-sulphur proteins. This could explain the reduction in sulphate detected as the sulphur cycle.

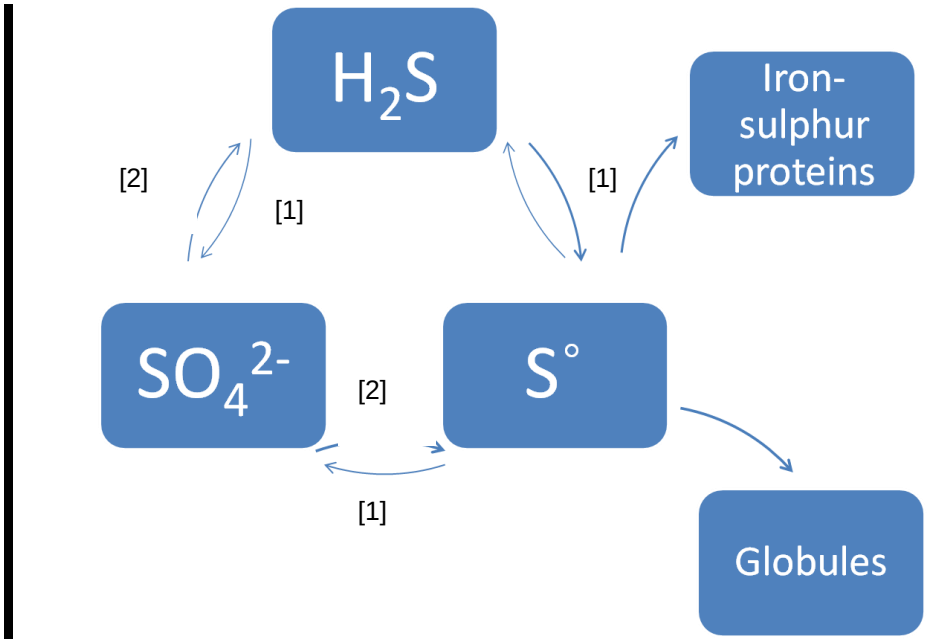

Figure 15. A conceptional model based on the sulphur cycle regarding the 90 d pioneer trials, the 30 d pioneer trial and the 30 d biofilm trial. The main reactions are indicated in bold arrows. Numbers [1] and [2] are represented by the microorganisms indicated in Table 6.

The main reaction drive will be to convert hydrogen sulfide and suphate to elemental sulphur by sulphur oxidizing bacteria. *Chlorobium* spp. will store the elemental sulphur in globules (Pott & Dahl, 1998) as in this manner safely remove the sulphur from the system. According to the NGS data, sulphate reducers were not detected and therefore the conversion of sulphur to sulphate and hydrogen sulphide would likely have been minimal.

Table 6. A summary of the main metabolic groups regarding the sulphur cycle present in the respective trials and representative micro-organisms thereof.

Metabolic group	Microbial genera	Reaction	Reference
1) Sulphur oxidizers	*Chlorobium* spp.	S°/ $H_2S \rightarrow SO_4^{2-}$	(Hell, 2008)
		S° → globules	(Pott & Dahl, 1998)
	Flavobacterium spp.	Thiosulphate/ $H_2S \rightarrow$ S°	(Hell, 2008)
		Iron-sulphur protein production	(Renger, 2008)
	Sphingobacterium spp	S°/ $H_2S \rightarrow SO_4^{2-}$	(Hell, 2008)
2) Sulphate reducers	*Desulfovirga* spp.	$SO_4^{2-} \rightarrow$ S°	(Tanaka *et al.*, 2000)
	Desulfuromonadales	$SO4^{2-} \rightarrow$ S°	
	Desulfovibrionales	$SO4^{2-} \rightarrow$ S°	

3.6.3.2. Sequence analyses of the 30 d pioneer and 30 d biofilm trials

Chlorobium spp. was dominant in the 30 d pioneer trial, followed by *Magnetospirillum* spp. and *Ornithobacterium* spp. The presence of *Magnetospirillum* spp. can be explained by the high concentrations of heavy metals present in AMD (Geremias *et al.*, 2003; Zhu *et al.*, 2010). Bacteria also detected in the 30 d pioneer trial included *Sphingobacteria* spp., *Flavobacteria* spp. (including *Ornithobacterium*), *Lentisphaeria* spp. (including *Victivallis* and *Lentisphaera*) Alphaproteobacteria including *Caulobacteraceae* spp., *Rhizobiales* spp., *Rhodopseudomonas, Salinarimonas, Rhizobium, Rhodospirillales* (including *Fodinicurvata* and *Magnetospirillum*), Epsilonproteobacteria including *Campylobacterales* spp., *Spirochaetes* spp., *Firmicutes* spp., *Clostridia* spp. (including *Ruminococcaceae, Sporobacterium* and *Lutispora*).

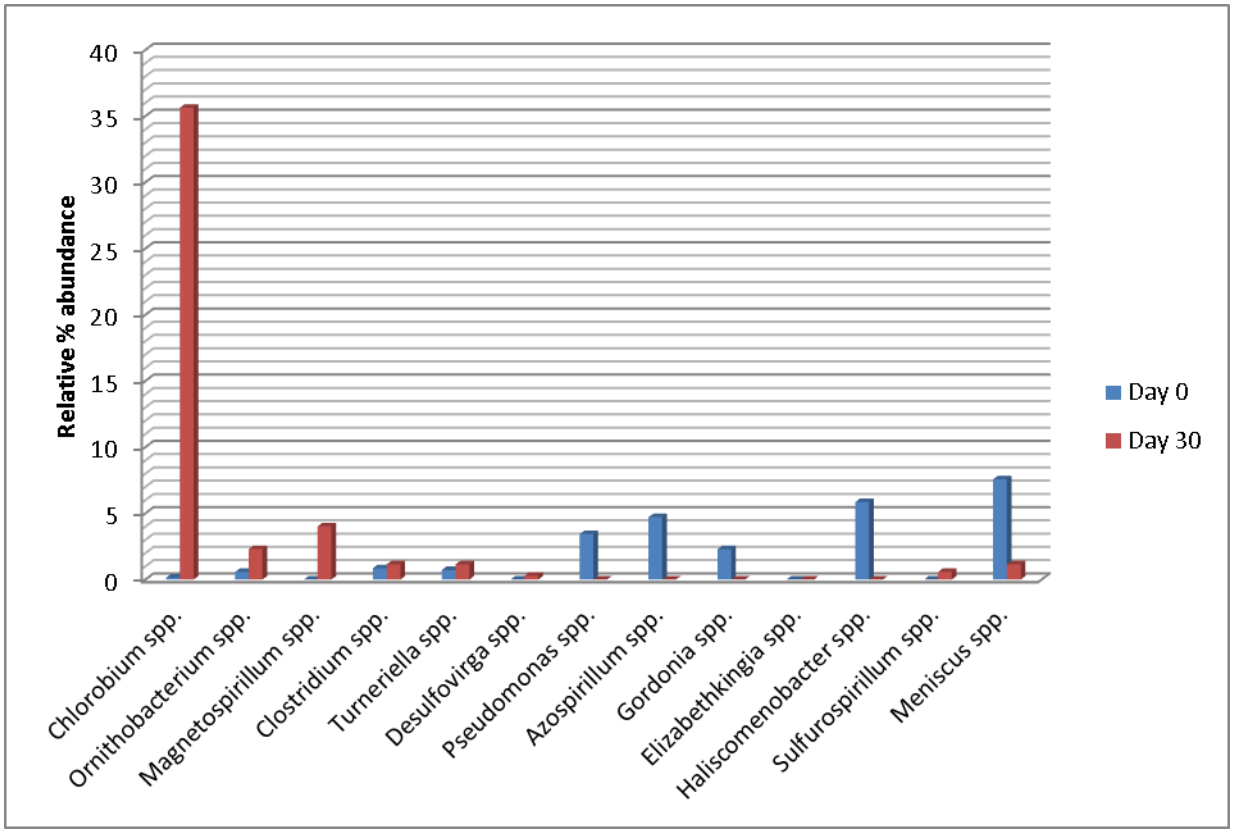

Figure 16. The microbial data obtained from the 30 d pioneer trial.

The sulphur cycle commencing in the 30 d pioneer trial may be depicted as seen in Figure 15. The microbial conversion of sulphate to hydrogen sulphide will commence and light will allow the reverse reaction of hydrogen sulphide back to sulphate (Mielczarek & McGrayne, 2000). Sulphur oxidizers including *Sphingobacteria* spp., *Flavobacteria* spp. and *Chlorobium* spp. converts sulphate to elemental sulphur under the anaerobic conditions (Brune, 1995; Frigaard & Dahl, 2008) and SRB present including *Desulfonatronovibrio* will reduce elemental sulphur to sulphate. However, as the number of sequences of SRB obtained is low (0.58 %) relative to the total number of sequences obtained by the current trial the reduction of elemental sulphur to sulphate should be lower compared to the oxidation of sulphate to elemental sulphur (the number of sulphur oxidizers relative to the total number of sequences obtained from this trial was about 36 %). Again elemental sulphur will be stored in globules by *Chlorobium* spp. (Pott & Dahl, 1998) or utilized during the production of iron/sulphur compounds by micro-organisms such as *Magnetospirillium* spp. (Mielczarek & McGrayne, 2000) which will increase sulphur removal from the system as it is a closed system. However, as the relative number of *Chlorobium* spp. is dominant at a relative percentage of 36 % the amount of sulphur stored in globules might be higher than the amount used for the production of iron/sulphur compounds.

The 30 d biofilm trial (Figure 17) had a higher presence of *Chlorobium* spp. (62 %) when compared to the 30 d pioneer trial (36 %) (Figure 16). This was expected as the treatment process already indicated a selection for *Chlorobium* spp. However the liquid contents did not display the same bright green appearance as the contents of the 90 d pioneer trials but also turned a dark brown colour similar to the 30 d pioneer trial (Figures 5 & 8). The cell suspensions of green sulphur bacteria are green or brown depending on the *Chlorobium* sp. present. Bacteriochlorophylls *c*, *d* and small amounts of *a* are present in the green species and bacteriochlorophyll *e* and small amounts of *a* are present in the brown species (Borrego & Garcia-Gil, 1995). Therefore it is possible that the brown green sulphur bacteria was present in the 30 pioneer and biofilm trials.

The microbial diversity of the 30 d biofilm trial also appeared to be higher than the 30 d pioneer trial that may be attributed to the established biofilm. This included species from the *Bacteroidetes* division (mainly *Prolixibacter*), *Bacteroidia* including *Alkaliflexus*, *Paludibacter* and *Petrimonas*, *Meniscus* and *Sphingobacteriaceae* from the *Sphingobacteria* division, the *Flavobacteria* division including *Ornithobacterium*, the division *Chlorobi*, *Elusimicrobia* spp., *Lentisphaeria* spp. including *Lentisphaera* and *Victivallis*, *Alphaproteobacteria* including *Caulobacteraceae* spp., *Rhodospirillales* spp., *Betaproteobacteria* including *Burkholderiales* spp. and *Rhodocyclales* spp., *Deltaproteobacteria* including *Desulfobacterales* spp., *Desulfovibrionales* spp. and *Geobacter*, *Epsilonproteobacteria* divisions including *Sulfurospirillum* and *Thioreductor*, *Gammaproteobacteria* including *Chelonobacter*, *Acinetobacter*, *Azomonas*, *Pseudomonas* and *Stenotrophomonas*. The *Spirochaetes* spp. present included *Brevinema* and *Turneriella* and other present micro-organisms include *Opitutus*, *Firmicutes*, *Clostridia* spp. including *Sedimentibacter*, *Anaerovorax*, *Johnsonella*, *Moryella*, *Sporobacterium*, *Peptococcus*, *Ruminococcaceae* spp. and *Negativicutes* spp.

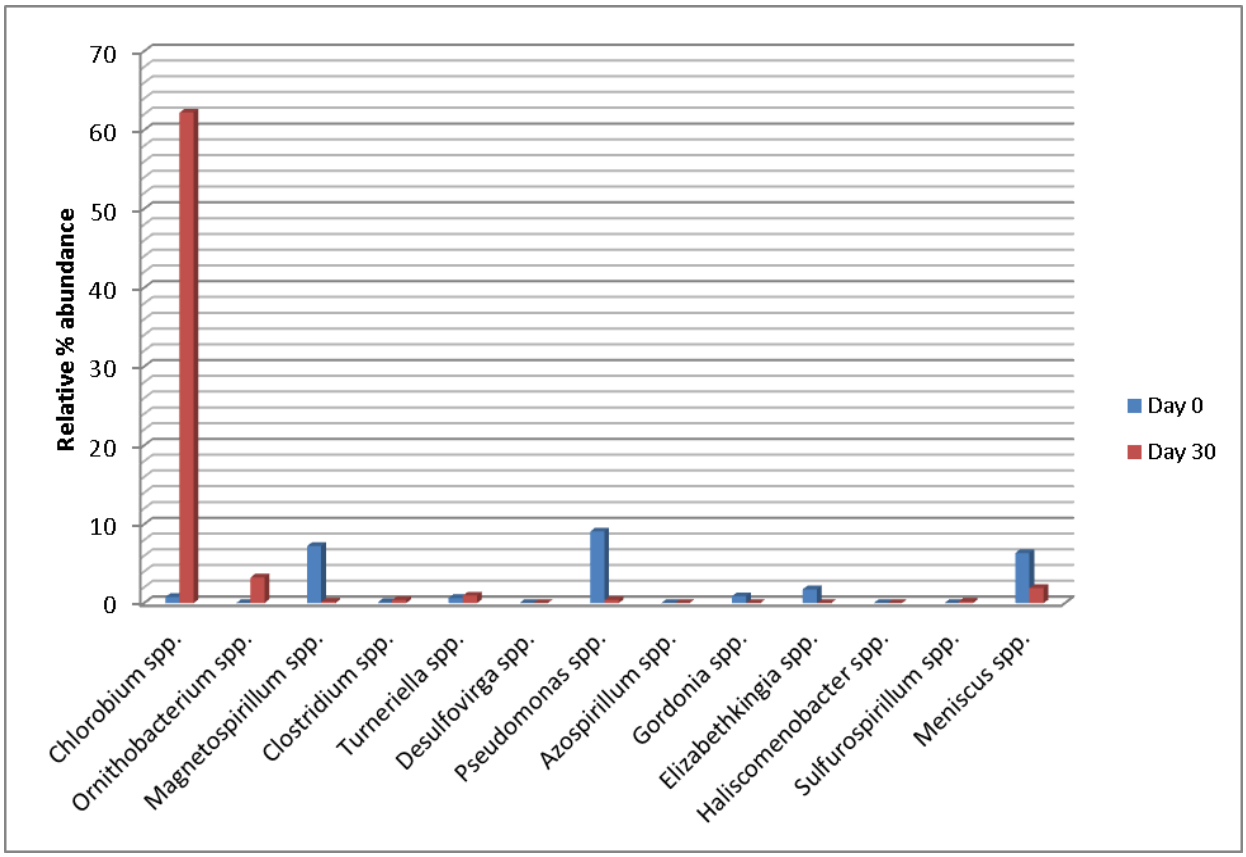

Figure 17. The microbial data obtained from the 30 d biofilm trial.

During the 30 d biofilm trial conversion of sulphate to elemental sulphur by sulphur oxidizers such as *Sphingobacteria* spp., *Flavobacteria* spp. and *Chlorobium* spp. under the anaerobic conditions (Brune, 1995; Frigaard & Dahl, 2008) and the conversion of sulphate to hydrogen sulphide as well as the reverse reaction (as previously described) under the light permitting conditions (Figure 15). Furthermore, sulphur will be stored in globules as previously described by *Chlorobium* spp. In contrast to the 30 d pioneer trial the conversion of sulphate to sulphur and the removal of sulphur by *Chlorobium* spp. should take place at a higher rate relative to the 30 d pioneer trial as the number of phototrophs detected in the 30 d biofilm trial is roughly 65 % in comparison to the roughly 37 % in the case of the 30 d pioneer trial.

3.6.4.3. Sequence analyses of the trial conducted at reduced incubation temperatures

Variation in microbial analysis was found in the duplicate trials at reduced incubation temperature. However the following section will discuss the two trials as a whole for the possible trend commencing in such a bioreactor under the relevant environmental circumstances.

The microbial analyses of the trial conducted at temperatures varying between 17°C and 19°C revealed more diversity compared to the 90 d pioneer trials. Species from the bacterial

divisions Alphaproteobacteria (*Caulobacterales*, *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales* and *Magnetospirillum*), Betaproteobacteria (*Burkholderiales* and *Rhodocyclales*), Deltaproteobacteria (including *Desulfovibrionales* and *Desulphuromonadales*) Epsilonproteobacteria including species from the genera *Campylobacterales* and *Nautiliales* Gammaproteobacteria including *Enterobacteriales* spp, *Xanthomonadales* spp, *Pseudomonas* spp., *Spirochaetes* spp, *Firmicutes* spp. and *Flavobacteria* spp. (including *Ornithobacterium* spp.) were detected.

The lack of sulfate removal can possibly be due to the contrast of sulphur oxidizing and sulfate reducing micro-organisms present as sulphur is likely continuously cycled without any netto sulfate removal reaction (Figure 19) (Table 6) (Widdel & Pfenning, 1981). Furthermore, the lower temperatures would likely reduce the growth and metabolic activity of the inhabiting bacteria and in that manner limit the removal of sulfate as temperature has been shown to significantly impact prokaryotic metabolism (Madigan *et al.*, 1997; Dijkstra *et al.*, 2011).

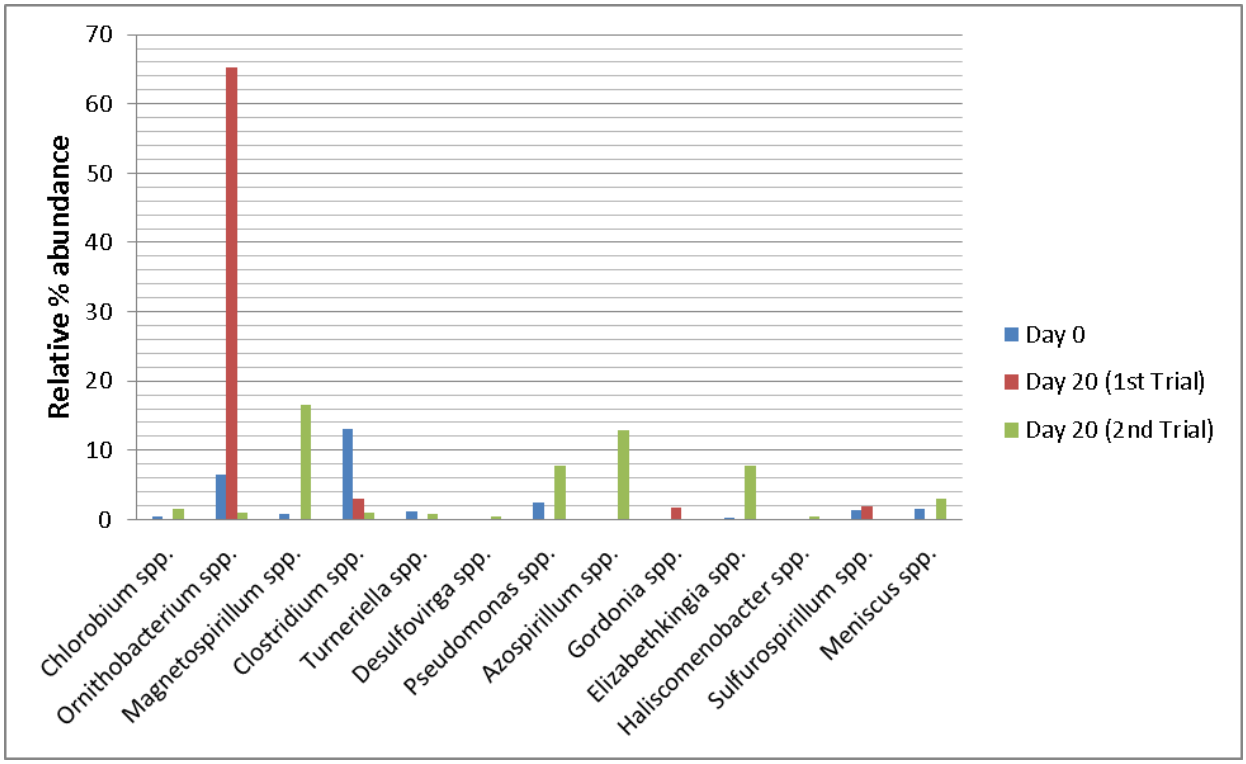

Figure 18. The microbial data obtained from the reduced incubation temperature trials.

Figure 19 depicts the conceptional model based on the microbial makeup of the reduced incubation temperature trials regarding the sulphate cycle.

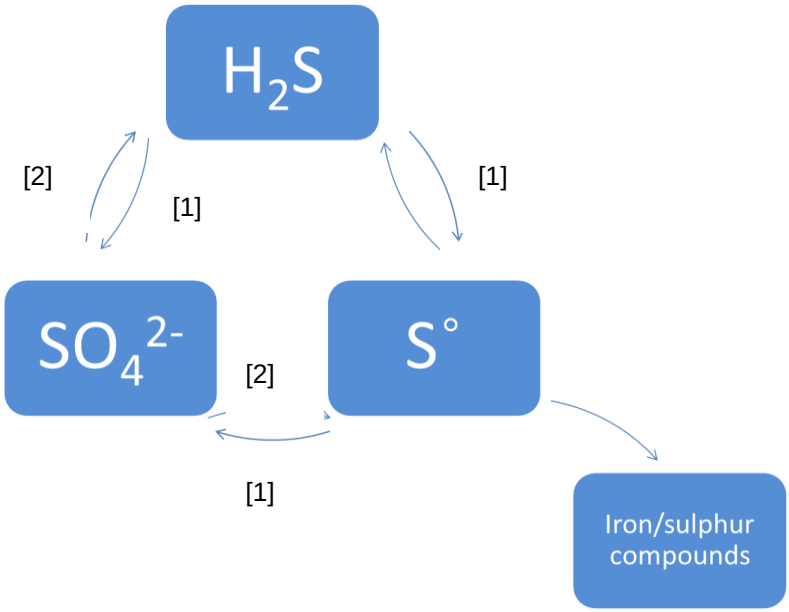

Figure 19. A schematic representation of the conceptional model derived from the gDNA sequencing data obtained from the trial conducted at temperatures between 17°C and 19°C. Numbers [1] and [2] are represented by the microorganisms indicated in Table 7.

Under the aerobic conditions at the beginning of the trial elemental sulphur will be converted to sulphate and hydrogen sulphide to elemental sulphur by members of the chemolithotrophic bacteria. As the environment become increasingly anaerobic, elemental sulphur will be converted to sulphate by anaerobic sulphur oxidizers (e.g. *Sphingobacteria* spp., *Flavobacteria* spp. and *Chlorobium* spp.) (Brune, 1995; Frigaard & Dahl, 2008). In contrast, SRB bacteria (e.g. *Desulfobacterales* and *Desulfovibrionales*) reduce elemental sulphur to hydrogen sulphide by dissimilative sulphate reduction (Barton & Fauque, 2009). Furthermore, some bacterial species will utilize iron in order to produce iron/sulphur compounds (Herskovitz, 1972; Neilands, 1981; Lill & Mühlenhoff, 2006).

An amount of sulphur might have been used for the microbial production of iron/sulphur compounds (by organisms such as *Magnetospirillum* spp.) as sulphur can become toxic to the cell and this is a safe way of storing sulphur (Mielczarek & McGrayne, 2000). The conversion of sulphate to hydrogen sulphide is microbial (Mielczarek & McGrayne, 2000). However, the conversion of hydrogen sulphide back to sulphate occurs in the presence of oxygen or light (Mielczarek & McGrayne, 2000). In this trial it would be the latter.

4. Conclusions

1. The use of a 1 L volume bioreactor and media with a similar COD and BOD to domestic wastewater sludge is successful in removing >96 % of sulphates and >85 % of the COD in 90 d.
2. An incubation period of 30 d reduced sulphates with 96 % and COD with 58 % and the presence of a biofilm in the bioreactors had no effect on COD and sulphate reduction.
3. Incubation temperatures at 17 - 19°C reduced sulphates and COD by only 12 % and 10 % respectively and is therefore not the ideal incubation temperature in the treatment of AMD with SDWWS.
4. Data of next generation sequencing showed that *Chlorobium* spp. was dominant in all the treatment trials except in the reduced temperature trials.
5. A few microbial species were not detected by next generation sequencing in the beginning of a trial although it was detected at the end of the treatment. This emphasise the importance of perspective when dealing with next generation sequencing technology as also stated by Shendure & Ji (2008).
6. Upscaling of the bioreactors in volume is needed to investigate treatment performance when treating larger volumes of AMD with domestic waste water sludge.
7. Trials using domestic wastewater sludge instead of SDWWS will determine whether a larger bacterial community would deliver different performance results than when using a 10 mL inoculum domestic wastewater sludge in 450 mL SDWWS.

Addendum A

Table 7. The microbial genera represented by the respective out's.

OUT	Size	Taxonomy
Otu0001	330	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0002	59	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0003	30	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0004	20	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0005	24	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0006	8	Bacteria(100);"Proteobacteria"(100);Alphaproteobacteria(100);Rhodospirillales(100);Rhodospirillaceae(100);Magnetospirillum(10
Otu0007	18	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0008	18	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0009	3	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu0010	13	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0011	12	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0012	13	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0013	18	Bacteria(100);"Spirochaetes"(100);Spirochaetes(100);Spirochaetales(100);Leptospiraceae(100);Turneriella(100);
Otu0014	14	Bacteria(100);"Proteobacteria"(100);Deltaproteobacteria(100);Syntrophobacterales(100);Syntrophobacteraceae(100);Desulfovirga
Otu0015	14	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0016	5	Bacteria(100);"Proteobacteria"(100);Gammaproteobacteria(100);Pseudomonadales(100);Pseudomonadaceae(100);Pseudomonas(1
Otu0020	3	Bacteria(100);"Proteobacteria"(100);Alphaproteobacteria(100);Rhodospirillales(100);Rhodospirillaceae(100);Azospirillum(100);
Otu0018	9	Bacteria(100);"Bacteroidetes"(100);"Sphingobacteria"(100);"Sphingobacteriales"(100);"Saprospiraceae"(100);Haliscomenobacter(
Otu0019	8	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0020	12	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0021	12	Bacteria(100);"Actinobacteria"(100);Actinobacteria(100);Actinomycetales(100);Nocardiaceae(100);Gordonia(100);

Otu0022	8	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0023	5	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Elizabethkingia(100);
Otu0024	13	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0025	13	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0030	7	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0027	10	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0028	9	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0029	10	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Elizabethkingia(100);
Otu0030	3	Bacteria(100);"Proteobacteria"(100);Alphaproteobacteria(100);Rhodospirillales(100);Rhodospirillaceae(100);Magnetospirillum(10
Otu0031	9	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0032	10	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0033	7	Bacteria(100);"Proteobacteria"(100);Epsilonproteobacteria(100);Campylobacterales(100);Campylobacteraceae(100);Sulfurospirill
Otu0034	3	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Elizabethkingia(100);
Otu0035	1	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu0036	4	Bacteria(100);"Proteobacteria"(100);Alphaproteobacteria(100);Rhodospirillales(100);Rhodospirillaceae(100);Magnetospirillum(10
Otu0037	5	Bacteria(100);"Proteobacteria"(100);Gammaproteobacteria(100);Pseudomonadales(100);Pseudomonadaceae(100);Pseudomonas(1
Otu0038	9	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0039	10	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0040	5	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu0041	3	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu0042	5	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0043	7	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0044	10	Bacteria(100);"Chlorobi"(100);"Chlorobia"(100);Chlorobiales(100);Chlorobiaceae(100);Chlorobium(100);
Otu0045	6	Bacteria(100);"Bacteroidetes"(100);"Sphingobacteria"(100);"Sphingobacteriales"(100);Cytophagaceae(100);Meniscus(100);
Otu0046	5	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0047	4	Bacteria(100);"Bacteroidetes"(100);"Bacteroidetes"_incertae_sedis(100);Prolixibacter(100);unclassified(100);unclassified(100);

Otu0048	3	Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Peptostreptococcaceae(100);Clostridium_XI(100);
Otu0049	7	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);
Otu0050	8	Bacteria(100);"Bacteroidetes"(100);Flavobacteria(100);"Flavobacteriales"(100);Flavobacteriaceae(100);Ornithobacterium(100);

References

Abolmaaty, A., Gu, W., Witkowsky, R., & Levin, R. E. (2007). The use of activated charcoal for the removal of PCR inhibitors from oyster samples.*Journal of microbiological methods*, 68(2), 349-352.

Almasi, A., & Pescod, M. B. (1996). Wastewater treatment mechanisms in anoxic stabilization ponds. *Water Science and Technology*, 33(7), 125-132

Barnes, H. L., & Romberger, S. B. (1968). In *Journal (Water Pollution Control Federation)*, 40(3), Part I, pp. 371-384. Water Environment Federation.

Barton, L.L., & Fauque, G.D. (2009). Biochemistry, Physiology and Biotechnology of Sulphate-Reducing Bacteria. *Advances in Applied Microbiology,* 68, 41-98.

Borrego, C.M., & Garcia-Gil, L.J. (1995). Rearrangement of light harvesting bacteriophyll homologues as a response of green sulphur bacteria to low light densities. *Photosynthesis Research,* 45, 21-30.

Brune, D. C. (1995). Isolation and characterization of sulphur globule proteins from Chromatium vinosum and Thiocapsa roseopersicina. *Archives of Microbiology,* 163(6), 391-9.

Burns, A. S., Pugh, C. W., Segid, Y. T., Behum, P. T., Lefticariu, L., & Bender, K. S. (2012). Performance and microbial community dynamics of a sulfate-reducing bioreactor treating coal generated acid mine drainage. *Biodegradation*, 2 (3), 415-29.

Center for disease control. (2003). Viewed 19 November 2014, from http://www.cdc.gov/brfss/annual_data/annual_data.htm

Characklis, W.G. (1990). 'Biofilm processes', in Characklis, W.G., Marshall, K.C. (eds.), Biofilms. Wiley, New York.

Collins, B. S., Sharitz, R. R., & Coughlin, D. P. (2005). Elemental composition of native wetland plants in constructed mesocosm treatment wetlands. *Bio-resource Technology*, 96(8), 937–948.

Cooper, P.F., & Sutton, P.M. (1983). Treatment of wastewaters using biological fluidized beds. *Chemical Engineering*, 392, 392.

Costello, C. (2003). Acid Mine Drainage: Innovative Treatment Technologies. *National Network of Environmental Management Studies*

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., & Lappin-Scott, H.M. (1995). Microbial biofilms. *Annual Review Microbiolology*, 49, 11-757.

Dijkstra, P., Thomas, S. C., Heinrich, P. L., Koch, G. W., Schwartz, E., & Hungate, B. A. (2011). Effect of temperature on metabolic activity of intact microbial communities: evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. *Soil Biology and Biochemistry*, 43(10), 2023-2031.

Druschel, G. K., Baker, B. J., Gihring, T. M., & Banfield, J. F. (2004). Acid mine drainage biogeochemistry at Iron Mountain, California. *Geochemical Transactions*, *5*, 13.

Frankin, R., Koevoets, W.A.A., van Gils, W.M.A., & van der Pas, A. (1992). Application of the Bio bed upflow fluidized bed process for anaerobic waste water treatment. *Water Science and Technol*ogy, 25, 373–382.

Frigaard, N. U., & Dahl, C. (2008). Sulfur metabolism in phototrophic sulfur bacteria. *Advances in microbial physiology*, 54, 103-200.

Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L., & Gonzalez, F. (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering*, 14(9), 997-1008.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Favere, V. T., Stolberg, J., Menezes, C. T. B., & Laranjeira, M. C. M. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental technology*, 24(12), 1509-1515.

Giesy Jr, J. P., & Briese, L. A. (1977). Metals associated with organic carbon extracted from Okefenokee Swamp water. *Chemical Geology*, 20, 109-120.

Greben, H. A., Bologo, H., & Maree, J. P. (2002). The effect of different parameters on the biological volumetric and specific sulphate removal rates. *Water SA*, 33-37.

Hell, R. (2008). Sulfur Metabolism in Phototrophic Organisms, Springer Science & Business Media, *Science*.

Herskovitz, T., Averill, B. A., Holm, R. H., Ibers, J. A., Phillips, W. D., & Weiher, J. F. (1972). Structure and properties of a synthetic analogue of bacterial iron-sulphur proteins. *Proceedings of the National Academy of Sciences*, 69(9), 2437-2441.

Hesham, A. E. L., Qi, R., & Yang, M. (2011). Comparison of bacterial community structures in two systems of a sewage treatment plant using PCR-DGGE analysis. *Journal of Environmental Sciences*, 23(12), 2049-2054.

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2009). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, 8(1), 15-25.

Hughes, T. A., Gray, N. F., & Guillamón, O. S. (2013). Removal of metals and acidity from acid mine drainage using liquid and dried digested sewage sludge and cattle slurry. *Mine Water and the Environment*, 32(2), 108-120.

James, G.A., Beaudette, L., & Costerton, J.W. (1995). Interspecies bacterial interactions in biofilms. *Journal of Industrial Microbiology*, 15, 257-302.

Johnson, D.B., & Hallberg, K.B. (2005). Acid mine drainage remediation options: a review. *Science of the Total Environment*, 338, 3–14.

Keller, J., Owens, C. T., Lai, J. C., & Devaud, L. L. (2005). The effects of 17 beta-estradiol and ethanol on zinc- or manganese-induced toxicity in SK–N–SH cells. *Neurochemistry International*, 46, 293–303.

Khan, S., Ahmad, I., Shah, M. T., Rehman, S., & Khaliq, A. (2009). Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management*, 90(11), 3451-3457.

Lazarova, V., & Manem, J. (1995). Biofilm characterization and activity analysis in water and wastewater treatment. *Water research*, 29(10), 2227-2245.

Lee, K. Y., Kim, K. W., & Kim, S. O. (2010). Geochemical and microbial effects on the mobilization of arsenic in mine tailing soils. *Environmental Geochemistry and Health*, 32, 31–44.

Lettinga, G., van Velsen, A.F.M., Homba, S.W., de Zeeuw, W., & Klapwijk, A. (1980). Use of the upflow sludge blanket reactor concept for biological wastewater treatment especially for anaerobic treatment. *Biotechnology and Bioengineering*, 22, 699–734.

Lill, R., & Mühlenhoff, U. (2006). Iron-sulphur protein biogenesis in eukaryotes: components and mechanisms. *Annual Review of Cell and Developmental Biology*, 22, 457-486.

Madigan, M. T., Martinko, J. M., Parker, J., & Brock, T. D. (1997). *Biology of microorganisms* (Vol. 985). Upper Saddle River, NJ: prentice hall.

Mielczarek, E.V., & McGrayne, S.B. (2000). Iron, Nature's Universal Element: Why People Need Iron And Animals Make Magnets. Diane Publishing Company, Nature.

McCauley, C. A., O'Sullivan, A. D., Milke, M. W., Weber, P. A., & Trumm, D. A. (2009). Sulfate and metal removal in bioreactors treating acid mine drainage dominated with iron and aluminum. *Water research*, 43(4), 961-970.

McKnight, D. M., Bencala, K. E., Zellweger, G. W., Aiken, G. R., Feder, G. L., & Thorn, K. A. (1992). Sorption of dissolved organic carbon by hydrous aluminum and iron oxides occurring at the confluence of Deer Creek with the Snake River, Summit County, Colorado. *Environmental Science & Technology*, 26(7), 1388-1396

Nairn, R. W., Hedin, R. S., & Watzlaf, G.R. (1992). *Generation of alkalinity in an anoxic limestone drain*. Proc. of the 9th Annual National Meeting of the American Society for Surface Mining and Reclamation, Duluth, Minnesota.

Neilands, J. B. (1981). Microbial iron compounds. *Annual review of biochemistry*, 50(1), 715-731.

Nicolella, C., Van Loosdrecht, M. C. M., & Heijnen, J. J. (2000). Wastewater treatment with particulate biofilm reactors. *Journal of biotechnology*, 80(1), 1-33.

Olem, H., & Unz, R.F. (1977). Acid mine drainage treatment with rotating biological contactors. *Biotechnology and Bioengineering*, 19(10), 1475-1491.

Poinapen, J., Ristow, N.E., Wentzel, M.C., Rose, P.D. & Ekama, G.A. Biological sulphate reduction of acid mine drainage using primary sewage sludge in a upflow anaerobic sludge bed (UASB) reactor. University of Cape Town, South Africa, unpublished date.

Pott, A.S., & Dahl, C. (1998). Sirohaem sulfite reductase and other proteins encoded by genes at the dsr locus of Chromatium vinosum are involved in the oxidation of intracellular sulphur. *Microbiology*, 144(7), 1881-1894.

Pulles, W., & Heath, R. (2009). The evolution of passive mine water treatment technology for sulphate removal, Golder Associates Africa (Pty) Ltd, PO Box 6001, Halfway House, 1685, South Africa. International Mine Water Conference.

Renger, G., & European Society Photobiology. (2008). Primary Processes of Photosynthesis: Principles and Apparatus (Comprehensive Series in Photochemical & Photobiological).

Sand, W., Gehrke, T., Jozsa, P. G., & Schippers, A. (2000). (Bio)chemistry of bacterial leaching – direct vs. indirect bioleaching. *Hydrometallurgy*, 59, 159–175.

Schloss, P.D., Gevers, D., & Westcott, S.L. (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS ONE. 6:e27310.

Shendure, J., & Ji, H. (2008). Next-generation DNA sequencing. *Nature biotechnology*, 30(10), 1135-1145.

Tanaka, K., Stackebrandt, E., Tohyama, S., & Eguchi, T. (2000). Desulfovirga adipica gen. nov., sp. nov., an adipate-degrading, gram-negative, sulfate-reducing bacterium. *International journal of systematic and evolutionary microbiology*, 50(2), 639-644.

Vieira, B.F., Do Couto, P.T., Sancinetti, G.P., Rodriquez, R. P., Klein, B., & Van Zyl, D. (2014). Biological treatment of acid mine drainage in batch reactors: Evaluation of initial pH and metal precipitation. *InfoMine.*

Widdel, F., & Pfennig, N. (1981). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. *Archives of Microbiology*, 129(5), 395-400.

Wuertz, S., Bishop, P. L., & Wilderer, P. A. (Eds.). (2003). *Biofilms in wastewater treatment: an interdisciplinary approach*. IWA Publishing.

Zhu, K., Pan, H., Li, J., Yu-Zhang, K., Zhang, S. D., Zhang, W. Y., Zhou, K., Ye, H., Pan, Y., Xiao, T., & Wu, L. F. (2010). Isolation and characterization of a marine magnetotactic spirillum axenic culture QH-2 from an intertidal zone of the China Sea. *Research in microbiology*, 161(4), 276-283.

Zhou, Q., Chen, Y., Yang, M., Li, W., & Deng, L. (2013). Enhanced bioremediation of heavy metal from effluent by sulfate-reducing bacteria with copper–iron bimetallic particles support. *Bioresource Technology*, 136, 413–417.

Chapter 5

General discussion

In the light of the urgent need for alternative AMD treatment methods the use of anaerobic microbial bioreactors in combination with synthetic domestic wastewater sludge (SDWWS) media showed promising results in terms of sulphate and COD removal.

Popular conventional AMD treatments include the use of constructed wetlands (Stottmeister *et al.,* 2003; Collins *et al.,* 2005; Khan *et al.,* 2009) and sulphate reducing bioreactors (Garcia *et al.,* 2001; Kappler & Dahl, 2001). The use of sulphate reducing bioreactors led to the investigation of suitable carbon sources (Zdyb, 1999; Gilbert, 2004) followed by the development of the integrated and managed passive treatment (IMPI) process (Pulles & Heath, 2009). However, these processes require long treatment times (Pulles & Heath, 2009) and large surface areas (Khan *et al.,* 2009).

Anaerobic domestic wastewater sludge is an identified suitable carbon source (Davison *et al.,* 1989; Strosnider *et al.,* 2011b-c; Hughes *et al.,* 2013; Strosnider *et al.,* 2013). However, the composition of domestic wastewater sludge is variable (Bhatti *et al.,* 1995; Tao *et al.,* 2012), hence using domestic wastewater sludge in laboratory experiments is challenging.

In Chapter 2 a synthetic domestic wastewater medium was formulated based on the COD and BOD of locally acquired anaerobic domestic wastewater sludge. The optimal ratio of AMD:SDWWS for the removal of sulphate and COD was tested. Anaerobic domestic wastewater sludge was used as inoculum and the trial was conducted at room temperature (≈23°C) in a dimly lit environment. The 1:1 ratio delivered the best results as it was able to reduce the COD with 85 % and the sulphate level with 98 %.

In Chapter 3 the microbial ecology was investigated in the treatment of AMD with SDWWS using different parameters. Medical drip bags were used as bioreactors and domestic waste water as the inoculum and incubation took place in a dimly lit environment at 25°C. COD and sulphate concentrations were determined and next generation sequencing (Iron torrent) in combination with Mothur 454 was used to determine the microbial species present in the beginning and end of each trial (Schloss *et al.,* 2011).

The first two trials took place over 90 d and sulphates and COD were removed at an average of 98 % and 85 % respectively. The liquid contents in the bioreactors turned bright green and TEM images showed the presence of green sulphur bacteria. *Chlorobium* spp. dominated in the two trials by relative percentages of 68 % and 76 % respectively. In the following trial (30 d pioneer trial) the minimum treatment time required for the removal of most of the sulphate

was determined. After 26 days the sulphate level was reduced by 96 % and reached a plateau. The COD reached a plateau at 60.8 % which could be due to the sulphate levels being reduced to levels insufficient for the responsible micro-organisms to use the COD. The liquid contents in the bioreactors changed to a dark brown colour. The 30 d pioneer trial selected for *Chlorobium* spp., *Ornithobacterium* spp. and *Magnetospirillum* spp. The presence of *Ornithobacterium* spp. and *Magnetospirillum* spp was to be expected as AMD characteristically contains heavy metals concentrations (Geremias *et al.,* 2003; Zhu *et al.,* 2010).

The impact of the presence of an established biofilm in the bioreactors under similar treatment conditions was studied in the treatment of AMD with SDWWS (30 d biofilm trial). Sulphate and COD removal were similar as in the 30 d pioneer trial at 96 % and 58 % respectively. This was unexpected as biofilms are popular for treating wastewaters for COD and sulphate content (Lazarova & Manem, 1995; Nicolella *et al.,* 2000; Wuertz *et al.,* 2003). Only a small percentage of the liquid content was in contact with the biofilm and this could be a reason why the COD and sulphate removal did not differ from the 30 d pioneer trial. *Chlorobium* spp, *Ornithobacterium* spp. and *Meniscus* spp. were dominant. When compared to the 30 d pioneer trial the microbial diversity of the 30 d biofilm appeared to be higher, which was to be expected as there was a biofilm present when the trial started. In both the 30 d trials a white precipitant was observed at the top of the bioreactors that consisted out of potassium, sodium, calcium, magnesium, sulphate and total organic carbon. The high concentration of sulphate also confirmed the dominance of *Chlorobium* spp. in the bioreactors as these species precipitate sulphur in the form of globules (Pott & Dahl, 1998). The liquid content was the same dark brown colour as in the 30 d pioneer trial. Green and brown green sulphur bacteria exist depending on the *Chlorobium* sp. present. Therefore it is anticipated that the green species were present in the 90 d pioneer trials and the brown species in the 30 d trials.

A conceptional model for the 90 d trials, 30 d pioneer trial and the 30 d biofilm trial regarding the sulphur cycle was depicted with phototrophic bacteria (*Chlorobium* spp.) driving the oxidation of sulphur to sulphate and the storage of sulphur in globules, thereby removing sulphur from the system, and sulphate reducing bacteria (SRB) (*Desulfobacterales* spp. and *Desulfovibrionales* spp.) reducing sulphate to sulphur. Some bacteria such as *Magnetospirillum* spp. might use iron present for the synthesis of iron-sulphur proteins which would remove iron and sulphur from the system.

In the last trial, the effect of a reduced incubation temperature (17°-19°C) was tested. After 20 days the trial was terminated because very low reduction in COD (12 %) and sulphate (10 %) was found. As micro-organisms have specific temperature requirements in order to grow and for metabolic reactions to take place it is predicted that the micro-organisms responsible for sulphate reduction did not grow at a reduced temperature (Greben *et al.*, 2002).

The major reactions regarding the sulphur cycle in the conceptional model for the trial at a reduced incubation temperature involved the conversion of sulphur to sulphate by phototrophic bacteria (*Chlorobium* spp.) and the conversion of sulphate to sulphur by SRB (*Desulfobacterales* spp. and *Desulfovibrionales* spp.). Some bacteria including *Magnetospirillum* spp. may utilize iron and sulphur in the production of iron-sulphur compounds. However, the ratio of phototrophic bacteria to SRB was smaller compared to the ratio of phototrophic bacteria and SRB during the remainder of the trials. Also, the presence of phototrophic bacteria itself was smaller relative to the total amount of bacterial sequences obtained from the reduced incubation temperature trials. Hence the removal of sulphur via the storage in globules by phototrophic bacteria could be reduced compared to the remainder of the trials.

General conclusions

- SDWWS simulating domestic wastewater sludge can be used as an alternative carbon source when investigating the treatment of AMD using anaerobic domestic wastewater sludge.
- Equal ratios of SDWWS and AMD incubated for 30 d at 25°C under anaerobic conditions using anaerobic domestic wastewater sludge as inoculum is adequate to reduce sulphates and COD to acceptable levels.
- *Chlorobium* spp. were the dominant microbial species involved in the removal of sulphates.

Future work

- Isolate and identify the *Chlorobium* spp., dominant in number of sequences obtained, on species level and evaluate as inoculum in the treatment of AMD with SDWWS in terms of minimum time required to reduce COD and sulphates.

- Isolate and identify the SRB species, dominant in number of sequences obtained, on species level and evaluate as inoculum as described above and then in combination with the above mentioned *Chlorobium* spp.
- Repeat above mentioned trials with *Magnetospirillum* spp. and *Ornithobacterium* spp. added to the inoculum. Determine the optimum ratio of the different species mentioned to obtain the desired sulphate reduction in minimum time.
- Up-scale the treatment process to investigate the treatment efficacy when treating larger volumes AMD and replace the SDWWS with real anaerobic waste water sludge.

References

Bhatti, Z. I., Furkukawa, K., & Fujita, M. (1995). Comparative composition and characteristics of methanogenic granular sludges treating industrial wastes under different conditions. *Journal of fermentation and bioengineering*, 79(3), 273-280.

Collins, B. S., Sharitz, R. R., & Coughlin, D. P. (2005). Elemental composition of native wetland plants in constructed mesocosm treatment wetlands. *Bio-resource Technology*, 96(8), 937–948.

Davison, W., Reynolds, C. S., Tipping, E., & Needham, R. F. (1989). Reclamation of acid waters using sewage sludge. *Environmental Pollution*, 57(3), 251-274.

Gadgil, A. (1998). Drinking water in developing countries. *Annual review of energy and the environment*, 23(1), 253-286.

Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L., & Gonzalez, F. (2001). Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Minerals Engineering*, 14(9), 997-1008.

Geremias, R., Pedrosa, R. C., Benassi, J. C., Favere, V. T., Stolberg, J., Menezes, C. T. B., & Laranjeira, M. C. M. (2003). Remediation of coal mining wastewaters using chitosan microspheres. *Environmental technology*, 24(12), 1509-1515.

Gilbert, O., De Pablo, J., Cortina, J.L., & Ayora, C. (2004). Chemical characterization of natural organic substrates for biological mitigation of acid mine drainage. *Water Research*, 38(19), 4186-4196.

Greben, H. A., Bologo, H., & Maree, J. P. (2002). The effect of different parameters on the biological volumetric and specific sulphate removal rates. *Water SA*, 33-37.

Hughes, T. A., Gray, N. F., & Guillamón, O. S. (2013). Removal of metals and acidity from acid mine drainage using liquid and dried digested sewage sludge and cattle slurry. *Mine Water and the Environment*, 32(2), 108-120.

Kappler, U., & Dahl, C. (2001). Enzymology and molecular biology of prokaryotic sulfite oxidation1. *FEMS microbiology letters*, 203(1), 1-9.

Khan, S., Ahmad, I., Shah, M. T., Rehman, S., & Khaliq, A. (2009). Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management*, 90(11), 3451-3457.

Lazarova, V., & Manem, J. (1995). Biofilm characterization and activity analysis in water and wastewater treatment. *Water Research*, 29(10), 2227-2245.

Nicolella, C., Van Loosdrecht, M. C. M., & Heijnen, J. J. (2000). Wastewater treatment with particulate biofilm reactors. *Journal of Biotechnology*, 80(1), 1-33.

Pott, A.S., & Dahl, C. (1998). Sirohaem sulfite reductase and other proteins encoded by genes at the dsr locus of Chromatium vinosum are involved in the oxidation of intracellular sulphur. *Microbiology*, 144(7), 1881-1894**.**

Pulles, W., & Heath, R. (2009). The evolution of passive mine water treatment technology for sulphate removal, Golder Associates Africa (Pty) Ltd, PO Box 6001, Halfway House, 1685, South Africa. International Mine Water Conference.

Roche Applied Science. Viewed 18 November 2014, from https://www.roche-applied-science.com.

Schloss, P.D., Gevers, D., & Westcott, S.L. (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS ONE. 6:e27310.

Stottmeister, U., Wießner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A., & Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances*, 22(1), 93-117.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011a). Novel passive co-treatment of acid mine drainage and municipal wastewater. *Journal of environmental quality*, 40(1), 206-213.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011b). Alkalinity generation in a novel multi-stage high-strength acid mine drainage and municipal wastewater passive co-treatment system. *Mine Water and the Environment*, 30(1), 47-53.

Strosnider, W. H., Winfrey, B. K., & Nairn, R. W. (2011c). Biochemical oxygen demand and nutrient processing in a novel multi-stage raw municipal wastewater and acid mine drainage passive co-treatment system. *Water research*, 45(3), 1079-1086.

Strosnider, W.H.I., Winfrey, B.K., Peera, R.A.M., & Nairn, R.W. (2013). Passive co-treatment of acid mine drainage and sewage: Anaerobic incubation reveals a regeneration technique and further treatment possibilities. *Ecological Engineering*, 61, 268– 273.

Tao, J., Wu, S., Sun, L., Tan, X., Yu, S., & Zhang, Z. (2012). Composition of Waste Sludge from Municipal Wastewater Treatment Plant. *Procedia Environmental Sciences*, 12, 964-971.

The European Bioinformatics Institute. Introduction to Next Generation Sequencing, viewed 18 November 2014, from http://www.ebi.ac.uk/.

Wuertz, S., Bishop, P. L., & Wilderer, P. A. (Eds.). (2003). *Biofilms in wastewater treatment: an interdisciplinary approach*. IWA Publishing.

Zdyb, L. (1999). Microbial sulfate reduction as a method of passive treatment of acid mine drainage using undefined carbon sources. University of Pretoria. South Africa. pp 67.

Zhu, K., Pan, H., Li, J., Yu-Zhang, K., Zhang, S. D., Zhang, W. Y., Zhou, K., Ye, H., Pan, Y., Xiao, T., & Wu, L. F. (2010). Isolation and characterization of a marine magnetotactic spirillum axenic culture QH-2 from an intertidal zone of the China Sea. *Research in Microbiology*, 161(4), 276-283.